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LABORATORY TECHNIQUES IN RABIES

THIRD EDITION

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PREFACE

During the 18 years that have elapsed since the publication of the first edition of Laboratory Techniques in Rabies *enormous progress has been made in improving methods of vaccine production and in developing new assay procedures and diagnostic techniques. These advances have been kept under constant review by meetings of research groups and of the WHO Expert Committee on Rabies, and in 1966 a second edition of the monograph was published incorporating a number of new chapters, while other chapters were revised and brought up to date.*

Since that time, the pace of research on viral techniques has quickened, bringing many advances that have greatly benefited work with the rabies virus. A third edition of the monograph has therefore become necessary. This edition includes some 20 new chapters in which are described techniques for the tissue culture of rabies virus and several new quantitative assay procedures applied to this virus, such as complement fixation and haemagglutination, as well as electron microscopy for structural and replicative studies. Many of the chapters retained from the previous edition have been extensively revised.

Particularly noteworthy is the inclusion of descriptions of the production and testing of recently developed vaccines, the production of antirabies immunoglobulin of human and animal origin, and the concentration and purification of the rabies virus. Most of the techniques described have already stood the test of time, but a few, such as the production of duck embryo and tissue-culture vaccines for use in man, should be considered provisional until more laboratories have had an opportunity to determine their practicability. Their inclusion was felt to be warranted by the wide interest that such techniques have aroused and the relative dearth of publications about them. It is therefore hoped that this revised edition will be useful not only to laboratories specialized in the production and testing of biological products for use in man and animals but also to workers interested in fundamental research on the rabies virus.

Proofs of this third edition of Laboratory Techniques in Rabies *were examined by the WHO Expert Committee on Rabies in December 1972 and a number of suggestions were made for minor changes in the text and for the inclusion of some additional material. Where this new material could not be incorporated in the existing text it has been added in the form of appendices at the end of the book.*

The World Health Organization gratefully acknowledges the collaboration of the many eminent specialists who have contributed to this volume.

PART I

GENERAL CONSIDERATIONS

CHAPTER 1

SAFETY PRECAUTIONS IN HANDLING RABIES VIRUS

M. M. KAPLAN[1]

Various publications are available describing the precautions to be taken in laboratories working with infective agents. The works listed at the end of this chapter include four manuals[2] that deal with problems associated with handling such agents, and Chapter 3 of this monograph is concerned particularly with the shipment and preparation of specimens from animals suspected of being rabid. The present chapter discusses the specific properties of the rabies virus that suggest the need for special precautions to protect personnel. It is noteworthy that apparently only one human death has occurred from a laboratory infection despite accidental needle punctures and other exposures to rabies virus during many decades of handling this virus in research, diagnostic, and vaccine production laboratories. This death was reported recently (1972) in a laboratory worker in Texas, USA, who was engaged in the preparation of vaccine and was exposed to aerosolized fixed virus from a blender. Nevertheless, the fear surrounding work involving rabies virus has resulted in relatively few laboratories doing badly needed research in this field. It is hoped that the following discussion will dispel undue concern, and will provide guidance on rational steps to be taken to avoid accidents and on how to deal with them when they do occur.

Properties of the Virus

The rabies virus belongs to the group of rhabdoviruses — enveloped, bullet-shaped, lipid-containing, and single-stranded RNA (Hummeler & Koprowski, 1969). It is sensitive to lipid solvents (soap solution, ether, chloroform, acetone), 45–70% ethanol, iodine preparations, and quaternary ammonium compounds (Kaplan et al., 1966). Other relevant properties are resistance to drying, to repeated freezing and thawing, relative stability at pH values between 5 and 10, and sensitivity to pasteurization temperatures and ultraviolet light. The nucleic acid is readily inactivated by β-propiolactone.

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[1] World Health Organization, Geneva, Switzerland.

[2] Forney, ed. (1968); Harris & Coleman, ed. (1963); Lennette & Schmidt, ed. (1969);

The virus is usually introduced by a bite wound, although penetration can occur through intact mucous membranes and the digestive tract (Fischman & Wards, 1968), but not through intact skin. Penetration of the virus into the cell in tissue culture systems occurs within 15 minutes, after which it can no longer be neutralized by specific antiserum (Kaplan et al., 1967). Airborne infection is possible in exceptional circumstances, for example, in caves harbouring large numbers of bats carrying the virus (Constantine, 1962). The virus travels centripetally, chiefly through the nervous system, although where large amounts of virus are injected blood-borne carriage is possible (Dean et al., 1963 ; Schindler, 1966 ; Baer et al., 1965). The virus may persist at the inoculation site for up to 18 days (Baer & Cleary, 1972), although a sharp drop in detectable virus occurs within a few hours (Habel, 1954; Schindler, 1961). In experimental animals the virus travels along the nerves centripetally at a rate of roughly 3 mm per hour, which is similar to the rate of spread of poliovirus. Progression of the virus seems to occur passively, through nerve-associated spaces, rather than in the perineural structures or the Schwann cells. In the central nervous system the virus is found in the grey matter and appears to multiply only in the neurones (Johnson, 1965). Spread of the virus centrifugally takes place along the nerves, but other routes may play a minor part.

There are marked differences between different strains of virus in their ability to infect, spread within the body, and produce disease. Laboratory strains of "fixed" virus used to produce vaccines, or employed in diagnostic and research procedures, have low pathogenic properties when inoculated peripherally in low doses. The Fermi-type vaccine (see Chapter 20), as well as the original Pasteur vaccine, contain residual, live, fixed virus. Such vaccines when properly prepared have been used safely, from the standpoint of innocuity for producing infection, for over 80 years. Accidents have occurred in man only when such fixed virus vaccines have not been properly incubated to reduce their virus content and large amounts of material of high titre have been injected into man (Para, 1965). Therefore, laboratory accidents involving slight puncture wounds when handling *fixed* virus are not very dangerous and need not cause worry if moderate treatment procedures are employed (see *Treatment of wounds* below). This includes all such strains as CVS, LEP, and HEP. The last two strains, as used in dry vaccines, have in fact been inoculated into hundreds of human beings without adverse effects.

Street virus, however, must be handled with respect, especially in the presence of hyaluronidase-containing saliva, despite apparent differences in the ability of different strains of street virus to infect through peripheral inoculation. This ability to infect and cause disease in animals is mainly a function of virus dosage, i.e., a threshold seems to exist that must be exceeded for disease to result. The susceptibility of man to small amounts of street rabies virus is apparently not as great as that of foxes and cattle, but since human infection has been known to occur even after relatively small puncture wounds, on the fingers for example, it is wise to consider all wounds with street virus as potentially very dangerous. In any event, all laboratory personnel should receive pre-exposure immunization (see below).

Laboratory Precautions [1]

All laboratories have their individual arrangements and consequent rules of discipline for handling infective materials. No attempt, therefore, is made here to cover the many possible variations of measures that could be used. Instead, general recommendations are given below for procedures that could be adapted to meet the major laboratory operations employed with rabies virus.

1. *Protective clothing.* Gross operations requiring the opening of skulls and spinal column, or those in which splintered material is encountered (broken glassware, bone), should be performed with thick protective gloves and goggles, and sleeved gowns. Rubber or strong plastic aprons that can easily be disinfected or discarded should also be worn. Close-fitting plastic or rubber gloves should be used when dealing with street virus inoculations into animals ; such precautions are not necessary, and are often a hindrance, when working with fixed or other attenuated strains of rabies virus in tissue culture and when performing titrations.

2. *Aerosols.* Since airborne rabies infection has been demonstrated (Constantine, 1962; Atanasiu, 1965; see also page 13), high-speed mixing and centrifugation procedures should be carried out in closed containers. Other operations that might cause aerosols (pipetting) should be carried out under a negative draught hood. Hoods and cubicles should be provided with ultraviolet lamps for disinfection when not in use. Pipetting by mouth should be prohibited.

3. *Disinfectants.* Quaternary ammonium disinfectants in 1 : 500 dilution, 45–70% alcohol, 1% soap solution, and 5–7% iodine solutions kill the rabies virus within one minute (Kaplan et al., 1966) and are indicated for the treatment of wounds (see below). For pipette receptacles a 1 : 1000 dilution of a quaternary ammonium compound, any iodine disinfectant with a residual available iodine of at least 1 : 10 000, or a 1% concentration of soapy water or detergent can be used. The solution should be autoclaved and discarded after each use. Hot soapy water or detergent can be used for swabbing floors and tables.

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into plastic or glass receptacles containing one of the disinfectants mentioned above. They should be autoclaved.

5. *Carcasses and animal tissues.* These are best disposed of in plastic bags and incinerated. Oral transmission of rabies in laboratory animals may occur when brains containing large amounts of virus are fed (Fischman & Wards, 1968). In countries at an early stage of economic development the carcasses of domesticated animals (sheep and goats) that have been used to prepare Fermi-type and Semple-type vaccines are sometimes employed for human consumption — after removing the head, viscera and vertebral column. In such instances, the meat should be thoroughly cooked before distribution.

Treatment of Wounds

All wounds should be washed immediately and thoroughly for several minutes with soap and water. This is perhaps the single most important procedure in preventing infection. The washing should be gentle in order to avoid further traumatization of the tissues. All soap should be removed before applying one of the chemical disinfectants mentioned under *Laboratory precautions.* Suturing of gaping wounds should be delayed for as long as possible (from several hours up to 3 days). Puncture wounds should be probed gently with an appropriate chemical disinfectant, taking care to minimize further trauma. Local infiltration of wounds with antirabies serum or immunoglobulin [1] (of human origin if available, to avoid serum reactions) should be employed in addition, if the wounded area so permits; this procedure is used particularly in puncture wounds where adequate cleansing and disinfection are not feasible. Additional measures should be employed where indicated (tetanus toxoid or antiserum).

Pre-Exposure Immunization

Various schedules have been tried for pre-exposure immunization of laboratory personnel. If time permits, the course of action recommended in the sixth report of the WHO Expert Committee on Rabies (1973) should be followed. The report states :

> Persons who run a high risk of repeated exposures, such as laboratory staff working with rabies virus, veterinarians, dog handlers, field naturalists, etc., should be protected by pre-exposure immunization. Such immunization consists of a short course of 3 injections of a potent antirabies vaccine, preferably free from paralytic factor, at 5–7-day intervals, followed by a booster injection of vaccine 1 month after the last dose. *The presence of virus-neutralizing antibody in vaccinated individuals should be ascertained on*

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[1] An international group of experts has recommended that the name "immunoglobulin" should be used in place of "gamma globulin" (*Immunochemistry*, 1972 **9**, 597).

> *a serum sample drawn 1 month after the booster injection.* If negative, booster doses should be repeated until antibodies become demonstrable. Further booster injections should be administered at intervals of 1–3 years as long as the exposed person remains at risk.
>
> Sufficient information is now available to permit the Committee to recommend that a person who, as a result of pre-exposure or postexposure treatment has demonstrated an antibody response in the past, be given 1 booster dose when subsequently exposed to rabies. Persons who have failed to develop neutralizing antibody after pre-exposure treatment should receive a full course of antirabies treatment when exposed or re-exposed. Persons who have previously received postexposure treatment with a proven immunogenic vaccine, but whose neutralizing antibody titre has not been determined, should receive 4 doses of a potent vaccine on days 0, 10, 20, and 90 after re-exposure.

Antibody responses have been obtained with as little as 2 doses of vaccine given at least 3 weeks apart, and this may be tried if there is a shortage of time. A booster dose should be given one to several months after the last inoculation. Any potent vaccine can be employed either intradermally (0.2 ml total dose in 2 locations, 0.1 ml at each) or subcutaneously or intramuscularly (2 ml of 5% tissue emulsion or its equivalent). It is important to determine, as stated in the above quotation, whether antibody has in fact resulted from the procedure. With pre-existing antibody and the known rapid recall response elicited by booster doses the recommended regime can be employed with reasonable confidence. The development of new, concentrated, and purified vaccines of tissue culture origin, with greatly increased antigenic content, will no doubt improve considerably the antibody levels now achieved with most conventional antirabies vaccines.

Exposed individuals who have not received pre-exposure immunization should be treated according to the recommendations made in the latest report of the WHO Expert Committee on Rabies (sixth report, 1973).

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CHAPTER 2

AN ASSESSMENT OF LABORATORY TECHNIQUES IN THE DIAGNOSIS AND PREVENTION OF RABIES AND IN RABIES RESEARCH

M. M. KAPLAN[1]

Few diseases cause as much anxiety as does rabies ; this applies both to the exposed individuals and to the health authorities responsible for its prevention and control. The laboratory occupies a central place in meeting the threat of rabies ; upon its verdict often depend both the decision whether or not to proceed with a long and painful course of treatment and the decision on the need to institute elaborate measures for controlling an epizootic in a community. The laboratory must also provide the necessary assurance that the biological products used for treatment and prevention in man and animals are efficient and safe.

The succeeding chapters in this manual describe selected but multiple methods for arriving at a diagnosis in the laboratory, for determining the acceptability of biological products in rabies prophylaxis, and for conducting rabies research. Most laboratory workers can decide for themselves whether one or other of the techniques given is within their competence, but often they are not aware of certain pitfalls and the limitations of particular methods. Also, a choice of procedures can ease the work and provide a decisive answer quickly. These considerations are partially covered in the relevant sections of this manual ; here they are reviewed and evaluated comparatively to serve as a possible guide for selecting procedures and for interpreting the results obtained.

The institution of treatment measures in exposed individuals, as recommended in the reports of the WHO Expert Committee on Rabies,[2] should never await the results of laboratory diagnosis. A laboratory diagnosis may be delayed for a variety of reasons and early treatment, both local and systemic, can be a critical factor in saving the life of the patient.

A laboratory report should be as clear and unequivocal as possible, and should stipulate exactly the procedures used. A positive test by any one of several procedures cancels negative reactions to the others. Thus, if a or attempted isolation of the rabies virus by animal inoculation gives negative results, the report should read " positive ". Where a doubtful result is obtained in any single test, recourse to the other tests available is essential in order to arrive at a definitive conclusion. Until this conclusion is reached, treatment should be continued as recommended in the above-mentioned report of the WHO Expert Committee on Rabies. Despite a completely negative laboratory report, circumstances may occasionally justify the initiation or continuation of treatment by the physician, e.g., suspicious clinical signs in the animal, or an unprovoked attack by an animal in an area where rabies is enzootic. Considerable experience has demonstrated, however, that a complete set of negative results obtained in a reliable diagnostic laboratory can be accepted with confidence, and that the treatment can be terminated or modified at that point.

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[1] World Health Organization, Geneva, Switzerland.

A. Diagnostic Procedures

1. Histopathology (chapters 3, 4 & 5)

The quickest and easiest procedure is the use of a suitable dye for the detection of Negri bodies. The Sellers, Giemsa and Mann stains are widely used and all of them are satisfactory. The Sellers stain combines simplicity with easy identification of the Negri bodies. With all the stains, care must be taken to distinguish the Negri bodies from inclusion bodies of other viral diseases and from artefacts. A competent laboratory worker can do this only after extensive training and supervision. A positive diagnosis based on the detection of Negri bodies can be made within half an hour after receipt of a specimen. If the reading is negative, the fluorescent-antibody technique (chapter 6) should be employed, if available, and the mouse inoculation test started (chapter 7). Rapid histological techniques are now available using frozen sections, but both this method and the classical method using paraffin-embedded sections are rapidly becoming obsolete now that rapid diagnosis can be achieved by means of the fluorescent-antibody technique ; they have value, however, in softened brain material and for research purposes where permanent mounts are desired.

The finding of specific inclusion bodies or rabies antigen (fluorescent-antibody technique) in the submaxillary salivary glands is of particular importance for treatment purposes. This examination should always be carried out because "animals do not bite with their brains" : the finding of rabies virus in the brain of an animal with none in the salivary glands removes some of the anxiety attendant upon treating an exposed person, although it does not entirely exclude the possibility that virus was being excreted at the time of the bite.

2. Animal inoculation (chapter 7)

The suckling mouse is the most susceptible animal for the inoculation test. While street virus has a variable incubation period of 7 to 18 days, and occasionally longer, rabies antigen can sometimes be detected by means of the fluorescent antibody test as early as the second day following inoculation. It is preferable to use 15–20 suckling mice or mice 3–5 weeks old for the inoculation test, so that 1 or 2 mice can be sacrificed each day, starting on the second day after inoculation ; this permits the use of the fluorescent-antibody technique and staining for Negri bodies for early detection of the virus. Negri bodies can sometimes be demonstrated as early as the third or fourth day after inoculation, before clinical signs begin.

Mouse inoculations should be employed in all cases where Negri bodies are not found, because in some laboratories positive results have been obtained by the mouse inoculation test in up to 20% of cases where Negri bodies were reported absent. In very experienced laboratories, the discrepancy may be much lower (less than 5%), and agreement between the fluorescent-antibody test and the mouse inoculation test approaches 100%.

3. Fluorescent-antibody (FA) test (chapter 6)

When properly performed, the " direct " FA test takes the lead over all others for speed and accuracy combined. Readings can be obtained within half an hour following receipt of the specimen, although for routine purposes a period of 2–4 hours or more is allowed for the fixation in cold acetone. The " indirect " FA test is useful for screening serum specimens for antibody, but this requires that the specific antiglobulin of the species concerned be conjugated with fluorescein isothiocyanate. Special FA equipment that can be used with ordinary light microscopes can now be purchased separately, so that the cost of adopting the FA technique has been much reduced and is now within the reach of most laboratories.

Apart from a satisfactory microscope, the two main requirements for success in using this technique are a well trained technician and conjugated serum of good quality. A minimum of several weeks' intensive training in a good laboratory is necessary even for a careful worker, but frequent confirmatory checks of positive and negative specimens using mouse inoculations should be carried out during the first year, or until complete confidence is gained as to the accuracy of the technique. After one year's experience most laboratories find over 99% agreement between the FA test and the mouse inoculation test (correlation of both positives and negatives). In the first and second year, however, some laboratories may miss up to 10% or even 20% of the positives with the FA technique, and for this reason the mouse-inoculation test must be used during this period on all antirabies serum (or immunoglobulin 1)) because of the difficulties involved; they should try to obtain the small supply usually required from a large central laboratory, or from reliable commercial sources. All batches of conjugated sera should be carefully tested on known positive and negative specimens, and *each* specimen tested should have a control as described in chapter 6. Only then can the diagnosis be accepted with complete confidence.

4. Virus neutralization index test (chapter 8)

This is the most accurate procedure for determining the specificity of the rabies virus. It should be combined with the fluorescent-antibody test for this purpose. In addition, this test can be used for detecting the presence and amount of antibody in a serum, although the serum dilution — constant virus technique is the preferred one in such cases. Since it is necessary to wait at least 14 days before reading the result, the virus neutralization test cannot be used for rapid diagnosis. (See also under " D. Research techniques ", p. 25, and chapter 9 for the plaque test.)

5. Complement-fixation test (chapter 10)

As classically employed (use of suspected brain as antigen), this test has had very limited usefulness for routine diagnosis because it gives less reliable results than the other tests described above. For research purposes, however, as described in chapter 10, the test has considerable merit.

6. Other tests[2]

Many other procedures can now be used for the isolation and detection of rabies virus in a research laboratory, but these tests are not suitable for routine diagnostic purposes. Isolation of rabies virus in various tissue culture systems (chapter 9) and electron microscopy of brain material (chapter 14) can be employed in rabies work, but the tests described previously are much easier to perform in diagnostic laboratories.

B. Production of Vaccine and Serum (Parts IV and VI)

1. Vaccine

The selection of a particular type of vaccine for production and use often poses a dilemma. This can be resolved if one keeps in mind the main purpose of a rabies vaccine: to protect against rabies. All other considerations are secondary, although admittedly important at times, e.g., paralytic accidents. The first principle is never to tolerate vaccines of low potency, regardless of other possible virtues (stability, absence of paralytic factor).

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[1] See footnote on p. 16.

[2] See also under " D. Research techniques ", page 25.

The laboratory should aim to produce the highest antigenic content in the lowest possible quantity of extraneous protein material. Improved vaccines for post-exposure treatment of man using purified and concentrated virions prepared in tissue culture (chapter 28) are now being tested in man, but such vaccines are costly and will not be available in most parts of the world for many years to come. Tissue culture vaccines produced from various cell substrates are being employed on a limited scale, and in laboratory tests they appear to be of comparable potency to nervous tissue vaccines (chapters 19–21) and duck-embryo vaccines (chapter 27), but they seem to be inferior to nervous tissue vaccines produced in suckling animals (chapters 22–24).

Avian-embryo vaccines (chapter 26), nervous tissue vaccines, and tissue-culture vaccines (chapter 29) are widely employed for pre-exposure prophylaxis in animals; all of them are effective for this purpose if they pass the relevant potency tests (see Part V).

The two main types of nervous tissue vaccine are Semple and Fermi, using heat and phenol for complete or partial inactivation respectively; the Fermi-type vaccine contains a slight residual amount of living fixed virus. Potent vaccines have been developed using brains of suckling animals (mouse, rat, rabbit), which have the advantage of being practically devoid of the paralytic factor. Many other types of vaccine are produced and widely used, e.g., ultraviolet-inactivated; duck-embryo (propiolactone-inactivated); vaccines inactivated with formaldehyde, chloroform (Kelser) or ether (Hempt); and dilutions of living fixed virus (Högyes, Pasteur). All these vaccines can now be produced in freeze-dried form, which is a great advantage from the standpoint of stability. Semple and Fermi type vaccines are the easiest to produce. Highly potent ultraviolet-inactivated vaccine is not difficult to produce once the apparatus has been properly adjusted. Difficulties are sometimes experienced in producing potent duck-embryo vaccine, as well as some of the other types (formol-, ether- and chloroform-inactivated). The Högyes, Fermi, and original Pasteur type vaccines are no longer widely employed and, in fact, are no longer recommended by the WHO Expert Committee on Rabies (see sixth report, 1973).

Regardless of the type of vaccine produced, in addition to a potency test all batches of vaccine should be subjected to the safety tests recommended, including those for the absence of contaminants and, with the Fermi-type vaccine, for ensuring only a low residual amount of live virus.

2. Serum and immunoglobulin (chapters 37-39)

Horses, mules, sheep, cattle, and rabbits — particularly the first three — are commonly employed for the preparation of antirabies serum, but in up to 20 % of cases adverse reactions to heterologous serum, often of a serious adverse reactions. The production of specific antirabies human immunoglobulin is a practicable procedure, although expensive. Sufficient stocks can be produced using the plasmaphoresis technique; this preparation avoids completely the adverse reactions resulting from heterologous serum.

C. Potency Tests (Part V)

1. Vaccine

Experience has shown that all batches of vaccine, regardless of the technique used, must be tested for potency, as this can vary even though the same method of production is employed routinely. The appropriate potency test described in Part V should be used. The value of the Habel test and its modifications and of the NIH antigen-extinction test using a reference vaccine (chapter 33) is well established for all vaccines except the living attenuated vaccines produced in chicken embryos for veterinary use. The latter require a different type of test (chapter 34). The rationale of potency tests is summarized in chapter 30.

It should be kept in mind that too great a quantitative significance should not be given to the protection index obtained either in the Habel potency test or in the NIH antigen-extinction test. In both cases, the index is calculated using either the Spearman-Kärber or the Reed & Muench method (see Appendix 1) and it should be considered as an approximation rather than as strictly quantitative. For example, a vaccine calculated to have an index of 12 000 in the Habel test cannot be said to be twelve times as potent as one found to have an index of 1000, the minimum acceptable figure for potency. Both the Habel test and the NIH test are designed to establish minimum rather than absolute values for potency.[1]

The stability of liquid-suspension vaccines is very variable. Unrefrigerated storage in hot climates or the freezing of a liquid phenolized suspension can completely destroy the potency of a vaccine. Dried vaccine containing too high a residual moisture content (over 2 %) is not usually stable. For vaccines produced and handled under certain local conditions, e.g., hot climates, frequent checks are necessary to determine that potency has been retained. The routine expiry dating of a vaccine cannot be depended upon where decentralization of treatment is employed, e.g., retention of vaccine stocks in rural treatment centres. The central laboratory should therefore recall specimens periodically from field stations to ensure that loss of potency has not occurred under their system of decentralization.

It should be noted that the NIH antigen-extinction test (chapter 33) can be performed on a volume dilution basis as well as on the basis of milligrams of nerve tissues. Volume dilution permits the present international reference (nervous tissue origin) vaccine to be used for testing tissue culture vaccines until such times as a more representative tissue culture reference vaccine can be developed.

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[1] A high potency value obtained in the Habel or NIH test should never be taken as a reason for diluting the vaccine or reducing its dosage.

The antibody binding test for potency of rabies vaccine (chapter 35) is promising but more work is required before it can be accepted as a routine test.

Nervous tissue vaccines that are claimed to be free of paralytic factor(s), e.g., vaccine prepared from brain of suckling animals (chapters 22–24), should be required to pass the test for freedom from neuroallergenic activity (chapter 36), even though this test does not provide infallible evidence of allergenic innocuity.

2. Serum

The potency test described in chapter 40 is essential to determine whether a particular batch of serum, or its globulin preparation, is acceptable with respect to its antibody content, based on international units.

D. Research techniques

The rabies virus is being increasingly used for basic studies in cell-virus relationships. This has led to the application of new techniques, some of which are described in Part III. Individual research laboratories often have their own preferences and introduce modifications to the usual procedures. The techniques described, therefore, can be considered as starting points for laboratories interested in research problems, with the rabies virus as a model.

Tissue culture of rabies virus (chapter 9) is not difficult. There is a wide choice of cell substrates, with almost any cell line supporting the growth of rabies virus in varying degrees. The plaque technique (chapter 9) is extremely useful for quantitating rabies virus and antibody, although some laboratories experience difficulty at first in employing this technique. Complement fixation, haemagglutination, haemagglutination-inhibition, and gel-diffusion techniques (chapters 10–13) are all applicable to rabies virus, although of these only the complement fixation technique has been extensively used. Electron microscopy, and purification and concentration of the rabies virus (chapters 14, 15, 17) are now widely employed in basic research ; the last two techniques are essential in the development and production of improved and highly antigenic vaccines. The immunoperoxidase reaction (Appendix 6) should prove useful as an early diagnostic tool, and the rapid tissue culture test for serum antibody (Appendix 5) is of great potential value where large numbers of serum specimens have to be screened for the presence of antibody. More work is required on both

PART II

ROUTINE LABORATORY DIAGNOSIS

CHAPTER 3

SHIPMENT OF SPECIMENS, AND TECHNIQUES FOR PREPARATION OF ANIMAL TISSUES

E. S. TIERKEL[1]

The animal suspected of being rabid should be captured and confined under observation for 10 days, if possible, and the disease should be allowed to progress until fatal termination. The premature killing of such animals will reduce the accuracy of the laboratory diagnosis, since the development of Negri bodies in the brain is directly related to the length of clinical illness in rabies. If circumstances necessitate the destruction of the animal, it should be shot through the heart, because shooting through the head will damage the brain and render it less useful for diagnosis. The use of chemical poisons is not recommended, since they may interfere with subsequent animal-inoculation tests.

Packing and Shipment of the Specimen

After decapitation of the animal in the field, the head should be cooled down promptly and kept cold. Wherever possible it should be delivered by messenger. If no messenger service is available, the head should be packed for shipment by express freight, via rail, air, or road. It should be put into a tin or other suitable watertight metal container, which should be closed tightly. This in turn should be put into a larger watertight metal container, cracked ice being packed between the inner and outer container (see Fig. 1 and 2). The package should be clearly labelled and shipped to the laboratory with utmost dispatch.

Heavy-gauge plastic bags and a shipping carton may also be used for sending heads to the laboratory. An inner bag (0.01 cm thick), measuring approximately 45 cm by 100 cm, should be sufficiently deep to allow the open end to be twisted and tied in a secure knot after the specimen has been placed inside. If the head has any sharp, protruding objects, such as shattered bone or porcupine quills, it should first be wrapped in several layers of newspaper, then placed into the bag. This knotted inner bag, containing the head, is then placed in a larger double-walled outer bag

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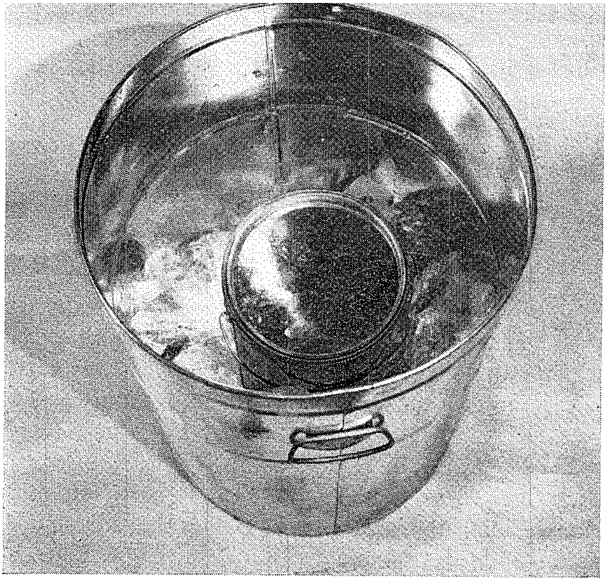
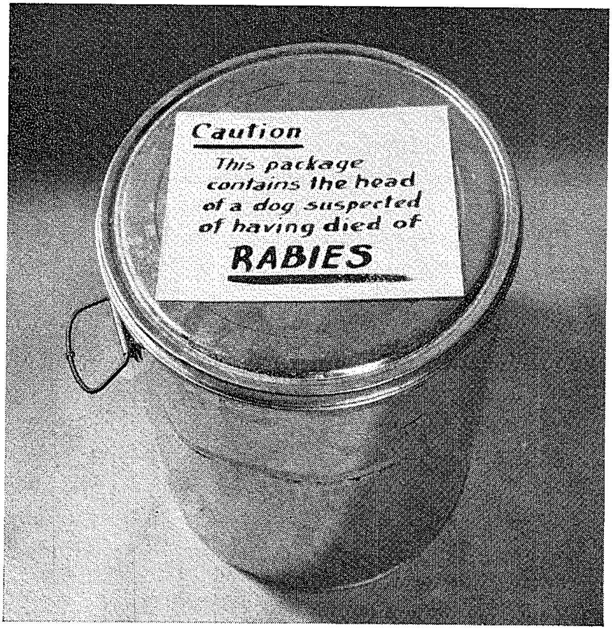
[1] [illegible]



By courtesy of United States Department of Health, Education and Welfare, Public Health Service, Communicable Disease Center (US DHEW-PHS-CDC)

FIG. 2. CONTAINER COVERED AND LABELLED

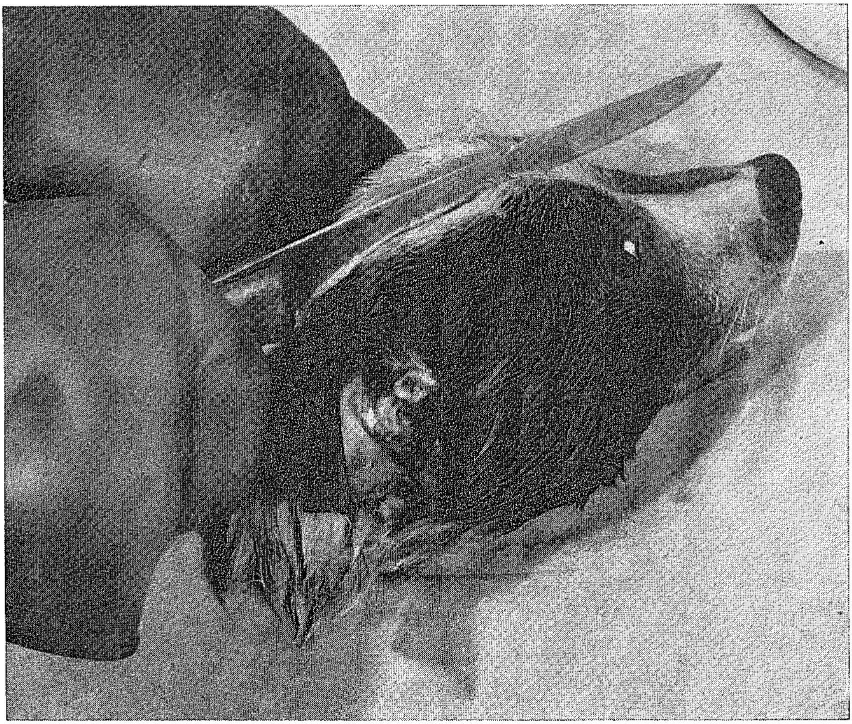

Caution
This package contains the head of a dog suspected of having died of
RABIES

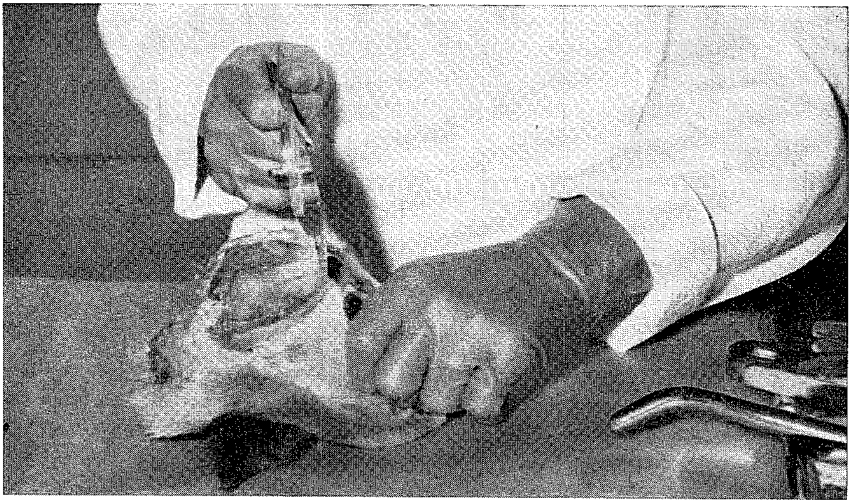
By courtesy of US DHEW-PHS-CDC

that is one-third full of crushed ice. The double-walled outer bag consists of two plastic bags, one inside the other, each approximately 0.008 cm thick, 60 cm wide and 120 cm deep. The specimen bag is then covered with additional crushed ice and the larger double outer bag is closed and sealed by twisting and knotting the open end. The entire package may now be placed in a corrugated cardboard carton measuring approximately 25 cm by 25 cm square and 45 cm deep. The carton should be sealed carefully with 8-cm wide self-adhesive plastic tape to make sure that the open-flap margins on the top and bottom of the carton are completely sealed.

NOTE : Although freezing the specimen and shipping it frozen in dry ice (solid carbon dioxide) or in nitrogen flasks will preserve the virus, it may prevent quick microscopic examination because of the time necessary for the head to thaw. Frozen portions of brain and salivary glands are easier to handle in the laboratory than are frozen entire heads. Immediately upon thawing, the tissues should be made ready for direct microscopic examination for Negri bodies, for the fluorescent antibody test, or for the mouse inoculation test.

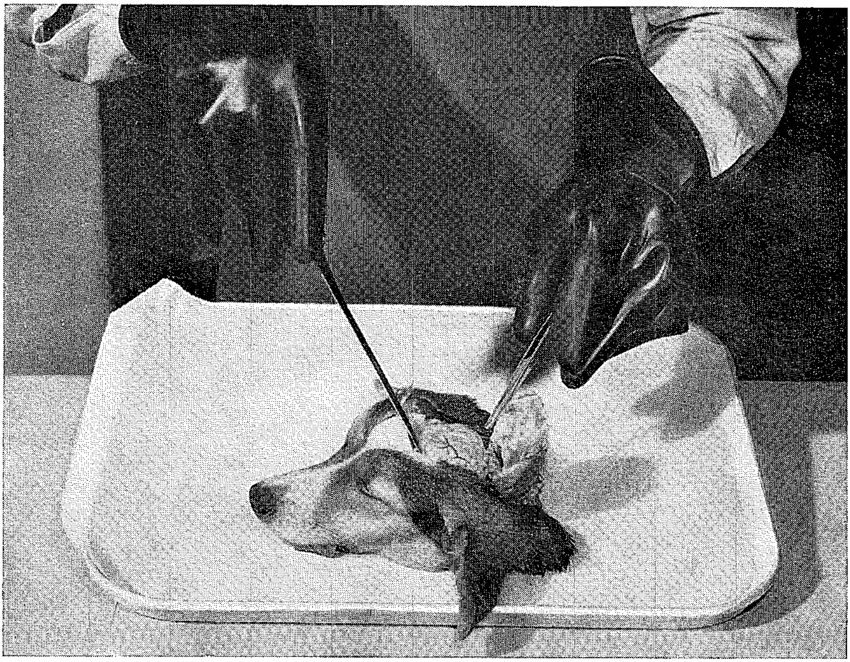
FIG. 3. INITIAL SKIN INCISION ALONG MIDLINE OF DOG'S HEAD





By courtesy of US DHEW-PHS-CDC

FIG. 5. CALVARIA REFLECTED



By courtesy of US DHEW-PHS- CDC

The following information is desirable when animal heads are received for examination: the species and breed of the animal; whether it was in contact with other animals; whether the animal died or was killed, and, if the latter, the means used for destroying it; whether the animal was confined and observed for an appropriate time before death, and, if so, for how long; symptoms of rabies, if any; history of vaccination against rabies.

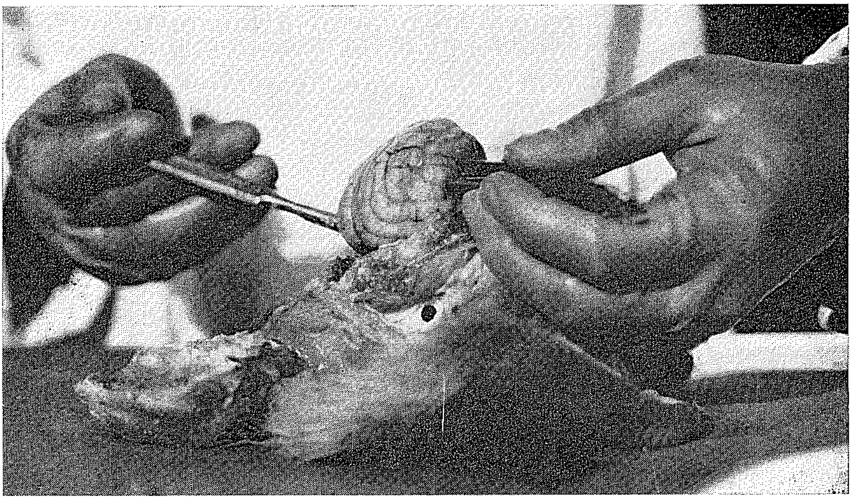
Removal of Animal Brain

All precautions, including careful operative technique and protection of the hands with heavy rubber autopsy-gloves, should be taken to prevent infection of persons opening animal heads.

The head should be held firmly on a solid table; proper immobilization may be effected by grasping it with a lion-jaw type of bone-holding forceps firmly applied to the maxilla. Improvised mechanical devices such as a carpenter's vice have often also been found effective.

With section knife or large scalpel a midline incision is made through the skin, fasciae, and muscles of the cranium, beginning anteriorly just above the level of the eyes and extending posteriorly to the base of the skull (see Fig. 3) The skin, fasciae, and temporal muscles are then dissected away from the cranium and reflected laterally, exposing the bone. The calvaria is then removed by means of a saw, bone-chisel, or butcher's cleaver. The sawing method is preferred by most laboratories. In this procedure a

FIG. 6. REMOVAL OF ENTIRE BRAIN



sterile surgical bone-saw is used to incise the bone of the skull on each side, beginning at the foramen magnum and then sawing anteriorly to the frontal bones. The longitudinal cuts are then joined by a transverse incision through the frontal plate just above the eyes (see Fig. 4). The calvaria is then lifted off with the aid of bone-cutting forceps and/or a bone-chisel (see Fig. 5).

With a fresh set of sterile instruments, the brain is now removed from the cranium. The meninges and the tentorium cerebelli, which separate the cerebrum and cerebellum, are dissected away with rat-tooth thumb forceps and either a scalpel or sharp-pointed scissors. Next, with a scalpel or scissors reaching back into the posterior portion of the brain, the brain is severed from its site by cutting into the medulla, cranial nerves, and anterior extension of the thalamus. The entire brain is then lifted out of the cranium on to a paper picnic plate or a large Petri dish (see Fig. 6).

If the body of the animal is available and no typical Negri bodies are found in the rapidly stained films, make a general post-mortem examination in an attempt to determine the reason for the illness or unusual behaviour of the animal. Wrap the remains in paper and ensure their incineration. Wash the table with 10% cresol solution. Sponge the gloves in the same kind of solution before removing them from the hands, and then sterilize them, with the instruments, by boiling.

It is always advisable to record the gross appearance of the brain, indicating especially the state of preservation, the presence or absence of adhesions and exudate, and whether the blood-vessels are injected. There are no gross pathological changes that can be regarded as diagnostic of rabies.

Even in the field, precautions should be taken to prevent bacterial contamination of the animal brain and sterile instruments should always be used for brain removal and dissection.

Removal of Salivary Glands

The examination of the salivary glands for the presence of virus is of obvious value in providing definitive evidence of whether or not a bite has entailed a risk. Presence of the infection in the central nervous system does not necessarily indicate infective saliva.

If virus is present in the salivary glands, the submaxillary (mandibular) glands will contain the most virus. For removal of the submaxillary salivary glands, the head is turned over so that the ventral aspect is facing upward. With an autopsy knife or scalpel, a midline incision is made in the skin covering the area between the rami of the mandible. The incision should begin at the mandibular lip and extend posteriorly to the neck. The skin is then reflected laterally, exposing the muscles and superficial soft tissues of the lower jaw.

The submaxillary salivary gland on each side is situated quite superficially and after reflecting the skin back it can be seen in the area of the posterior border of the mandible behind and below the superficial submaxillary lymph glands, with which the salivary glands should not be confused (see Fig. 7 and 8). The submaxillary salivary gland is elliptical in shape, about 5 cm long and 3 cm wide ; it is rounded in outline, greyish-yellow or orange in colour, and covered by a fibrous capsule.

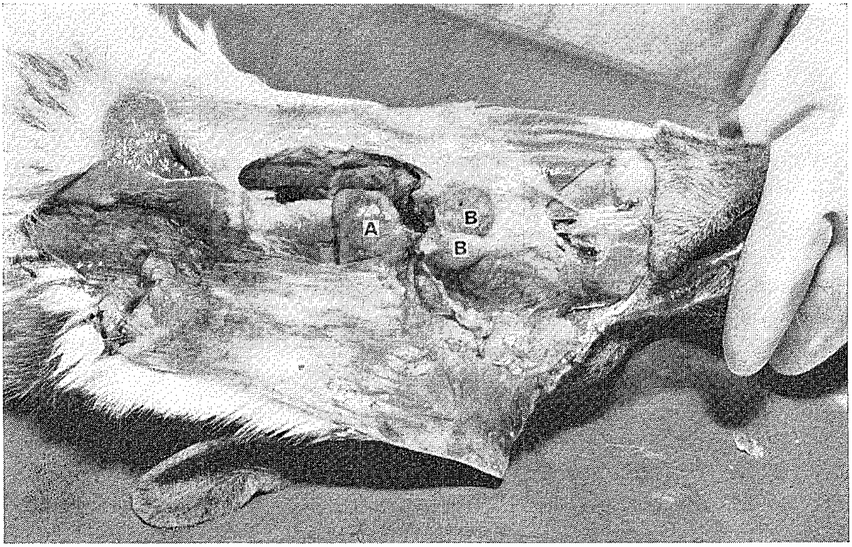
With a fresh sterile scalpel and rat-tooth thumb forceps, the gland on each side is dissected away from its surrounding tissues and placed in a Petri dish.

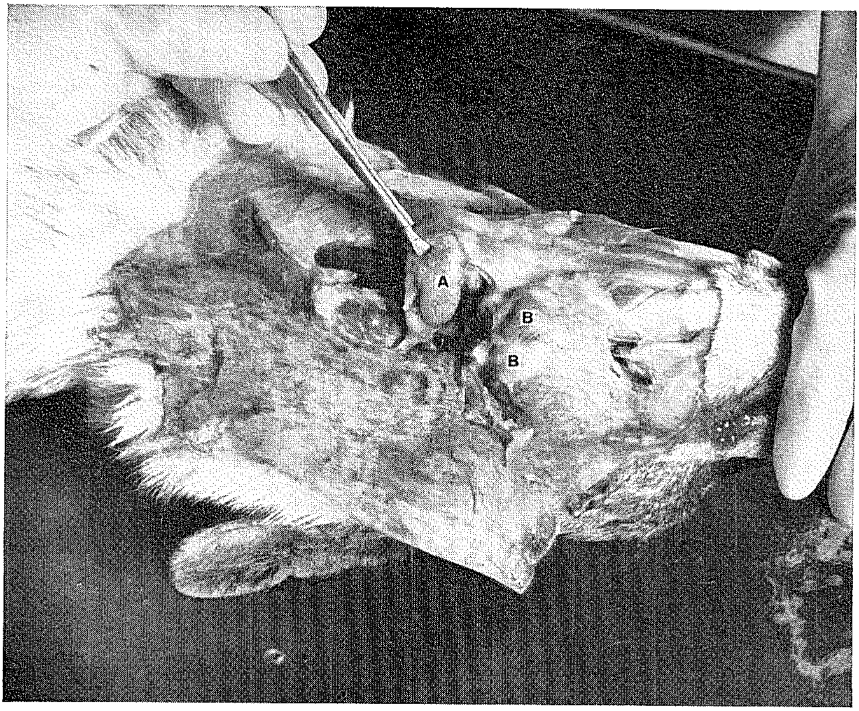
A small portion of each salivary gland is cut with sterile scissors and the portions are pooled in a single mortar for preparation of the salivary-gland suspension for the mouse inoculation test (see chapter 7, page 85). The test pieces should be weighed before emulsification for calculation of the concentration of the tissue suspension (see page 62 for gasserian ganglion).

Glycerolated Specimens

When facilities for animal inoculation of Negri-negative or doubtful animal brains suspected of rabies infection are not at hand, it is possible to ship portions of brain or salivary gland to a laboratory that performs animal inoculation tests.

FIG. 7. SKIN REFLECTED AND SUBCUTANEOUS TISSUE REMOVED, EXPOSING SUBMAXILLARY SALIVARY GLAND





A Submaxillary salivary gland
B Submaxillary lymph glands

A solution of sterile 50% glycerol-saline is prepared by adding equal parts of chemically pure glycerol to physiological salt solution. This solution may then be placed in small bottles or jars with screw tops, autoclaved, and stored at room temperature.

Fairly large portions of brain or salivary gland, at least about the size of a mouse brain (0.3 g or more), should be selected in order to give the laboratory worker sufficient material with which to work. Portions of the brain should include parts of the hippocampus, cerebellum, and cerebral cortex from each side. A portion of the medulla or brain stem should also be included. Place the tissue pieces in a bottle of prepared 50% glycerol-saline. Rabies virus, if present, will be preserved during shipment, and no refrigeration is required.

Glycerolated portions of brain do not usually produce satisfactory impressions on a slide unless washed thoroughly in saline, since it is difficult to make the glycerolated brain adhere to a slide. This is true whether the tissue impression is to be used for Negri body staining or for the fluorescent antibody test. For this reason, if further examination of brain smears of the questionable specimen is desired, smears may first be made from the unpreserved brain and stained, and the slides included in the shipment along with the glycerolated brain. Alternatively, brain smears may be made and the *unstained* slides plunged immediately into chemically pure methanol (acetone-free) for fixation, and then removed and dried at room temperature without blotting. These unstained, fixed smears can then be stained at the laboratory to which they are referred.

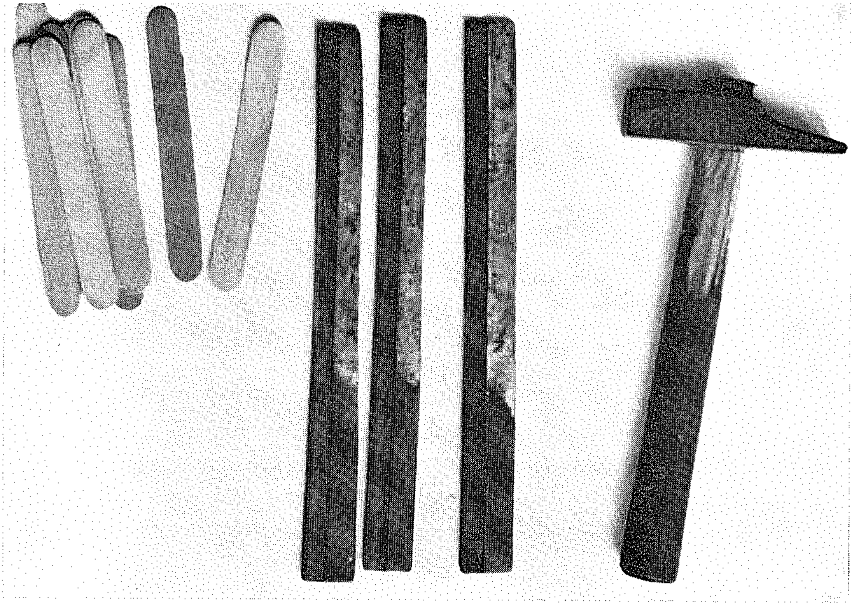
Upon their receipt in the laboratory, glycerolated tissue specimens should be immediately removed from the shipping bottle and placed in a sterile Petri dish. The pieces of tissue are then thoroughly washed in physiological saline solution by continual addition of the solution, gentle rotary agitation of the Petri dish, and discarding of the liquid. This is repeated several times. At this point it is as well to try making a smear preparation of the washed brain-tissue specimens for Negri-body staining and microscopic examination. The quality of the smear will depend on the amount of glycerol washed from the brain tissue. The remaining specimens are now ready for emulsification and mouse inoculation. For grinding salivary glands in a mortar a small amount of sterile sand or alundum is essential, but these materials are superfluous for making brain suspensions.

Note : It is advisable to retain in glycerol solution some unused portions of all animal-tissue specimens until the animal inoculation test either has been reported positive for rabies or has run its course (at least 21 days) and been reported negative for rabies.

Annex

REMOVAL OF ANIMAL BRAINS ACCORDING TO METHOD USED IN BERNE *

Conventional methods for opening the skulls of larger animals and removing their brains for rabies examination are time-consuming. At the Institute of Veterinary Bacteriology and Parasitology in Berne, we have adopted a routine procedure that permits the rapid removal of brain material, a particular advantage when large numbers of animals are received for examination. The technique is easily acquired by laboratory personnel and is as safe as other methods if properly executed. The method is used with minor modifications in many veterinary diagnostic laboratories throughout Europe.



A. Heavy hammer. B. Strong cutting blades (sharpened two-thirds of the distance from one end as used for trimming horses' hooves).

FIG. 10. SEVERANCE OF THE HEAD FROM THE NECK

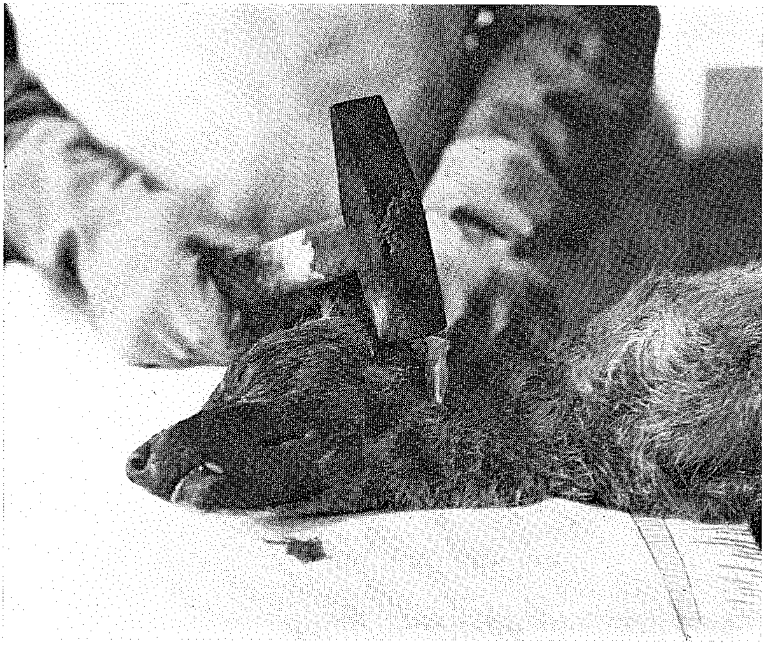


FIG. 11. SPLITTING THE SKULL

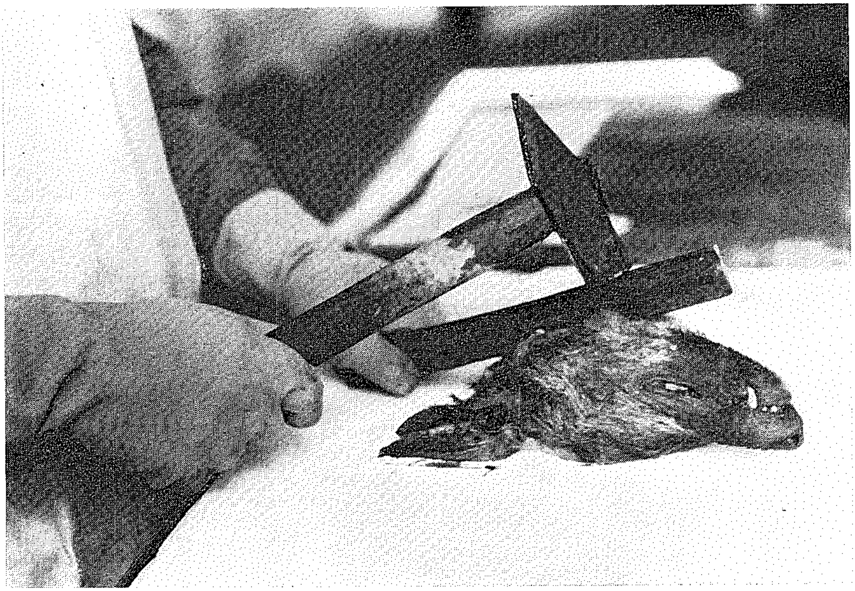
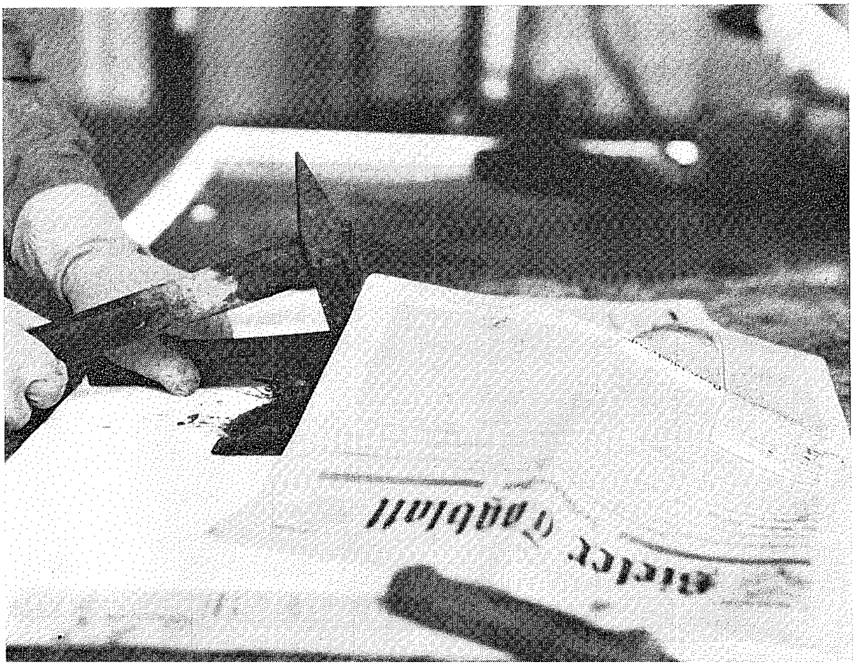
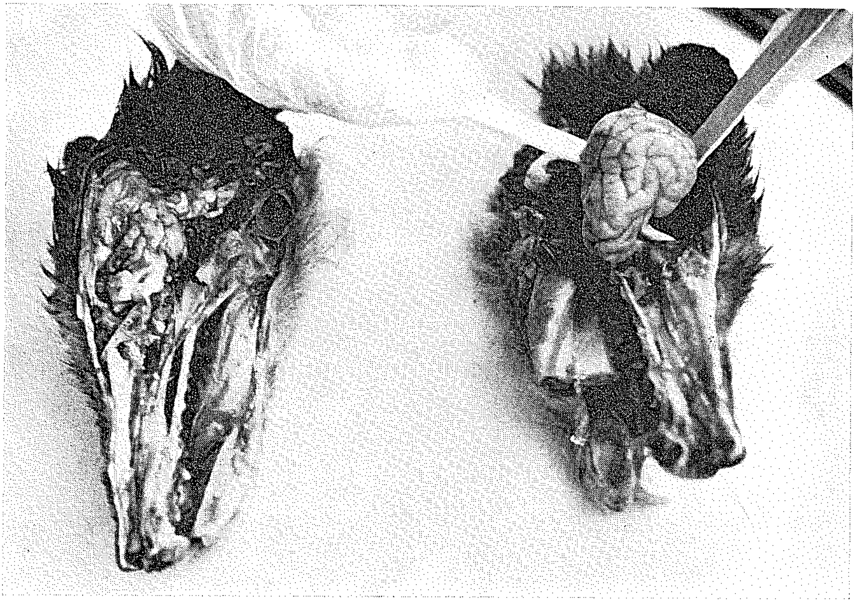


FIG. 12. USE OF NEWSPAPER TO PREVENT SPREAD OF DROPLETS





The tools necessary to open the skull are shown in Fig. 9. The techniques for splitting the head and removing the brain are illustrated in Fig. 10 to 13. The head is severed from the neck between the occiput and the atlas (Fig. 10). No vertebrae should be left in the skull. Both during this operation and during the subsequent splitting of the head the blade should be covered with sheets of newspaper to avoid the spilling of droplets (Fig. 12). The head should be placed either on the dorsal or the ventral surface, depending on the species of animal, and split completely into two halves along the sagittal plane (Fig. 11). It may facilitate this procedure if the skin and tissues are first cut with scissors. After the skull has been split, the brain is removed from each half with the aid of wooden spatulas, allowing it to fall under its own weight (Fig. 13).

CHAPTER 4

RAPID MICROSCOPIC EXAMINATION FOR NEGRI BODIES AND PREPARATION OF SPECIMENS FOR BIOLOGICAL TEST

E. S. TIERKEL[1]

The techniques employed in the laboratory diagnosis of rabies should embrace optimum conditions of accuracy, speed, and economy. The method employing microscopic examination for Negri bodies, using the simple application of brain tissue to a slide and the Sellers technique for staining (see Annex, page 52), fulfils these requirements.

It has been found that Negri bodies, when present, are most readily demonstrated in Ammon's horn (hippocampus major) of the brain and also in the pyramidal cells of the cerebral cortex and Purkinje's cells of the cerebellum; they are found to a much more limited extent in the neurons of the thalamus, pons, medulla, spinal cord, and sensory ganglia.

Dissection of the Brain

A very simple operation is required to expose Ammon's horn, which is generally the best area for demonstration of Negri bodies in most species of rabid animals. With a pair of sterile scissors, a longitudinal incision is made into the dorsal surface of each cerebral hemisphere, about 2 cm lateral to the longitudinal fissure or midline of the brain (see Fig. 1). The incision is made from the region of the occipital pole of the hemisphere and is extended forward for 3–5 cm and downward through the grey matter, and then completely through the white matter until a narrow space, the lateral ventricle, is reached. The opening is then widened by spreading the incised hemisphere, and Ammon's horn will be revealed as a semi-cylindrical, white, glistening body bulging laterally from the ventricle floor (see Fig. 2 and 3). It has a spiral contour and, on cross-section, a characteristically rolled surface.

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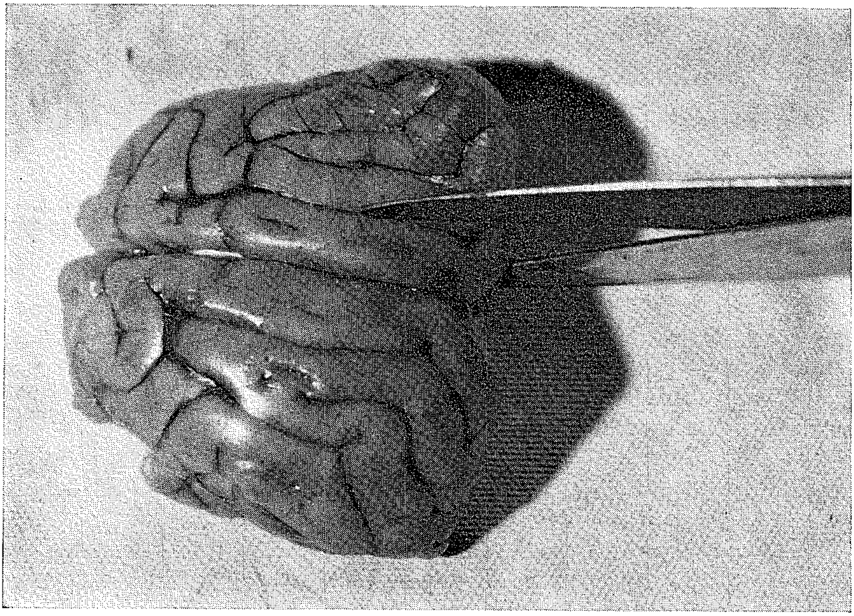
[1] [illegible]



By courtesy of United States Department of Health, Education and Welfare, Public Health Service, Communicable Disease Center (US DHEW-PHS-CDC)

Preparation of Slide

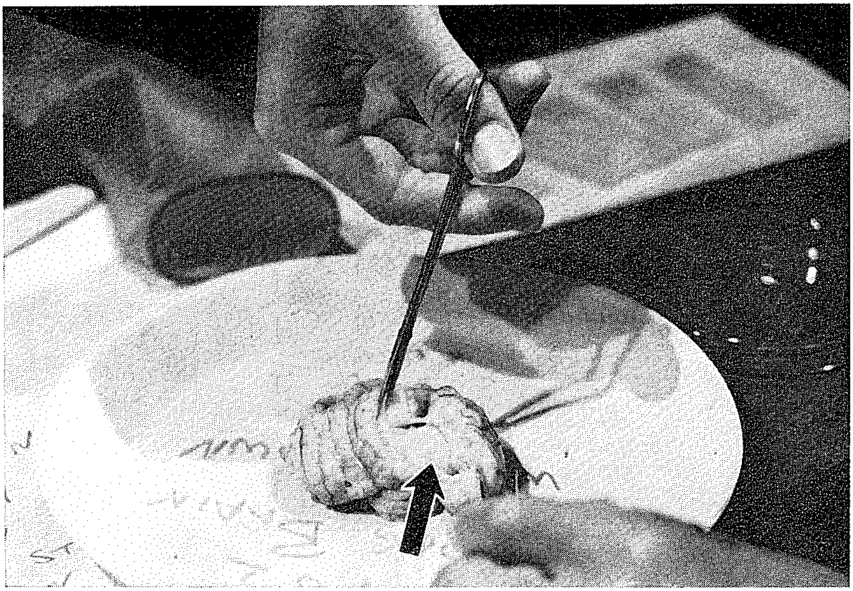
Slides should be made first from Ammon's horn, then from the cerebral cortex, and finally from the cerebellum. Samples (at least 6) should be taken from these 3 areas on each side of the brain and examined microscopically before the brain is reported Negri-negative. It is always wise to select another area from each hippocampus for good measure.

The following 3 methods of applying fresh brain tissue to slides can be recommended :

Impression method

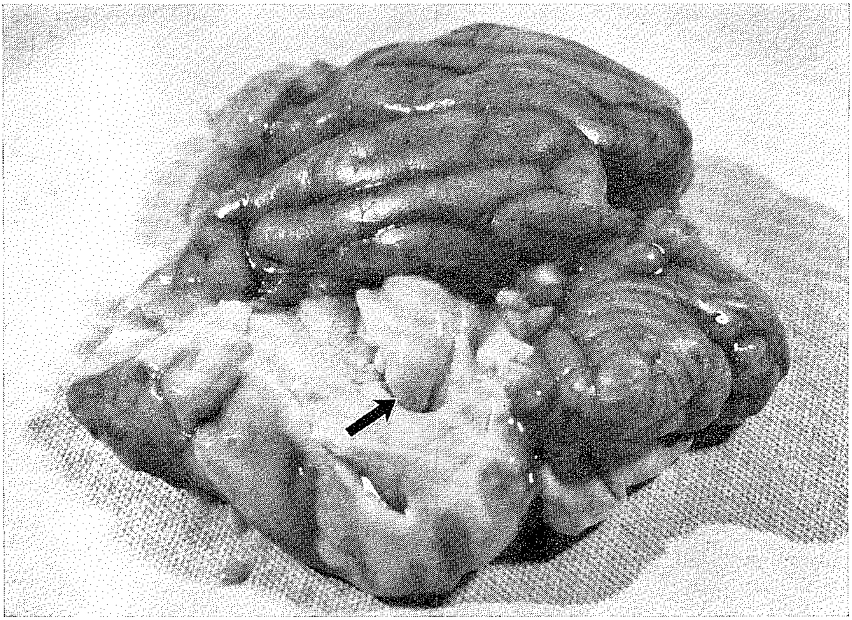
With a pair of scissors, small transverse sections (2–3 mm in thickness) of brain tissue (Ammon's horn, cerebrum, or cerebellum) are cut and placed on clean blotting-paper or a wooden tongue-depressor, cut surface facing upward (see Fig. 4 and 5). A clean microscope slide is then touched against the cut surface of the section and pressed gently downwards with just enough pressure exerted to create a slight spread of the exposed

FIG. 2. INCISION TO LATERAL VENTRICLE, SHOWING AMMON'S HORN *



By courtesy of US DHEW-PHS-CDC

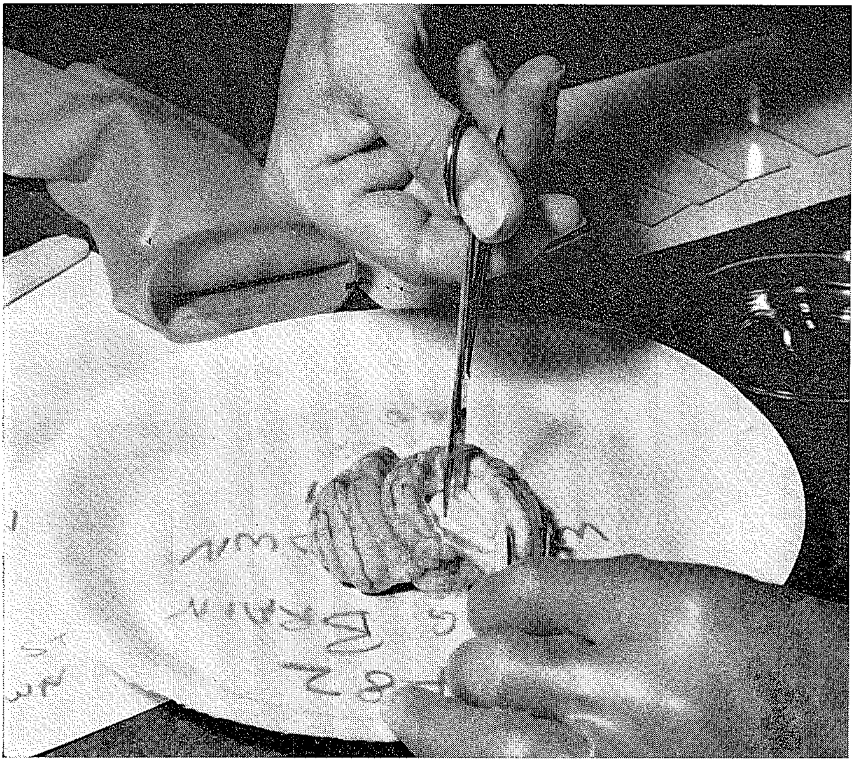
FIG. 3. CLOSE-UP OF AMMON'S HORN * BULGING FROM FLOOR OF LATERAL VENTRICLE



By courtesy of US DHEW-PHS-CDC

tion, 3–4 impressions can be made on one slide (see Fig. 6 and 7). *While still moist*, the slide is flooded with Sellers stain (see Fig. 8), allowed to remain for a few seconds, rinsed under the tap, and dried at room temperature without blotting. The preparation is then ready for examination. The impression may be examined directly under oil, or covered with a cover-slip mounted in balsam. This method is preferred over others because a maximum amount of nerve tissue can be concentrated in a small area with a minimum amount of cellular damage.

FIG. 4. TAKING OF SPECIMEN FROM AMMON'S HORN FOR SLIDE PREPARATIONS

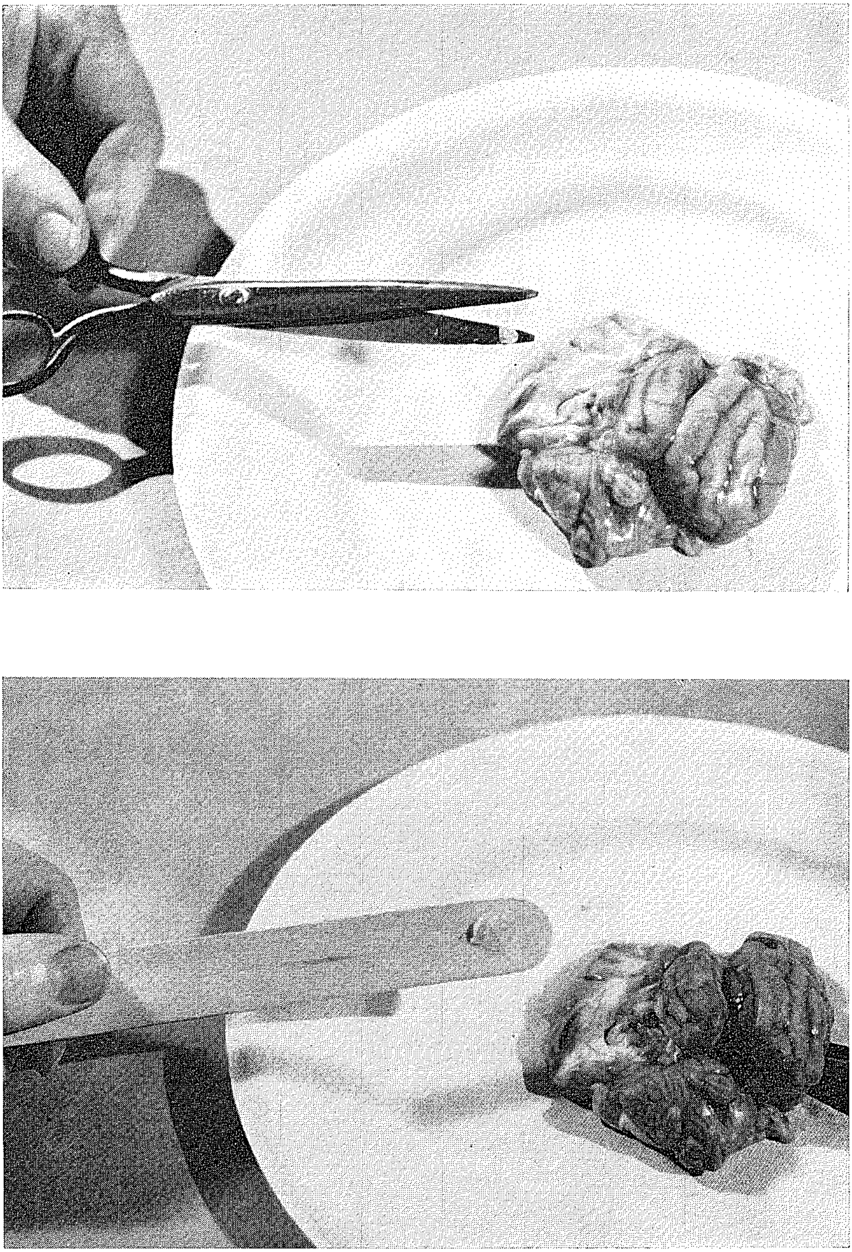


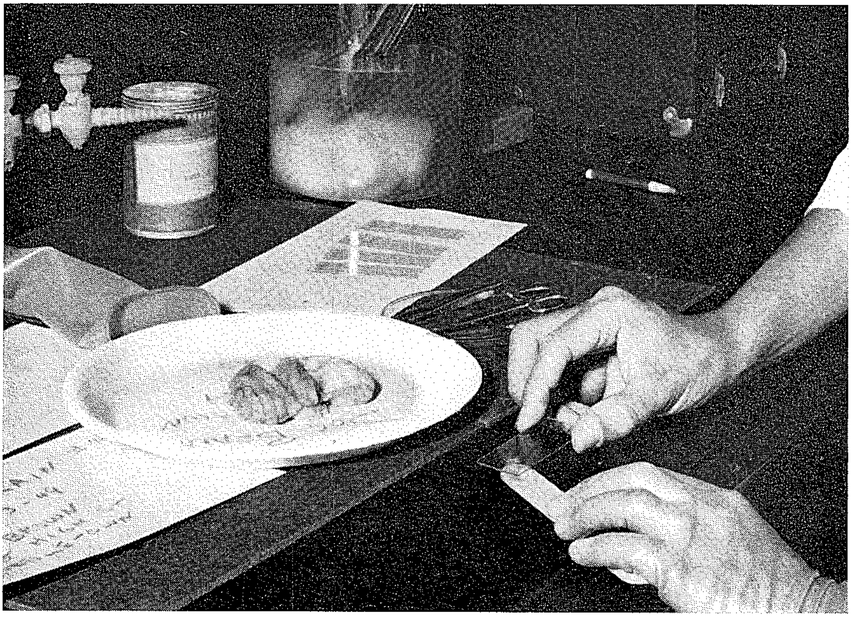
By courtesy of US DHEW-PHS-CDC

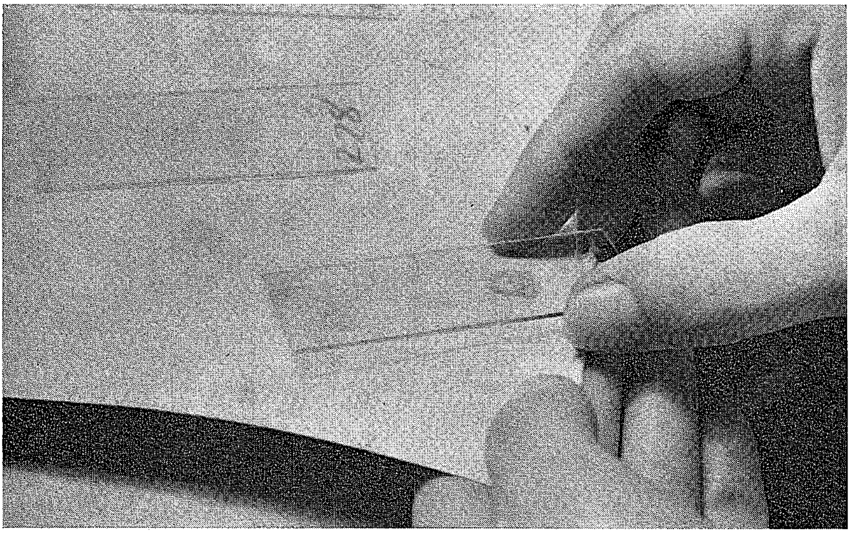
Smear method

The spread-smear method consists of placing a very small section of brain tissue on one end of the slide. Another slide is used to crush the section of tissue against the first slide and is then drawn across the length

FIG. 5. TRANSFER OF SPECIMEN TO WOODEN SPATULA BEFORE MAKING IMPRESSIONS

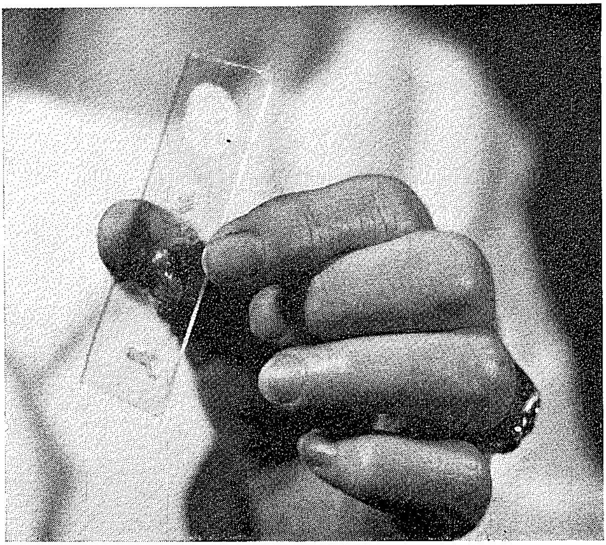






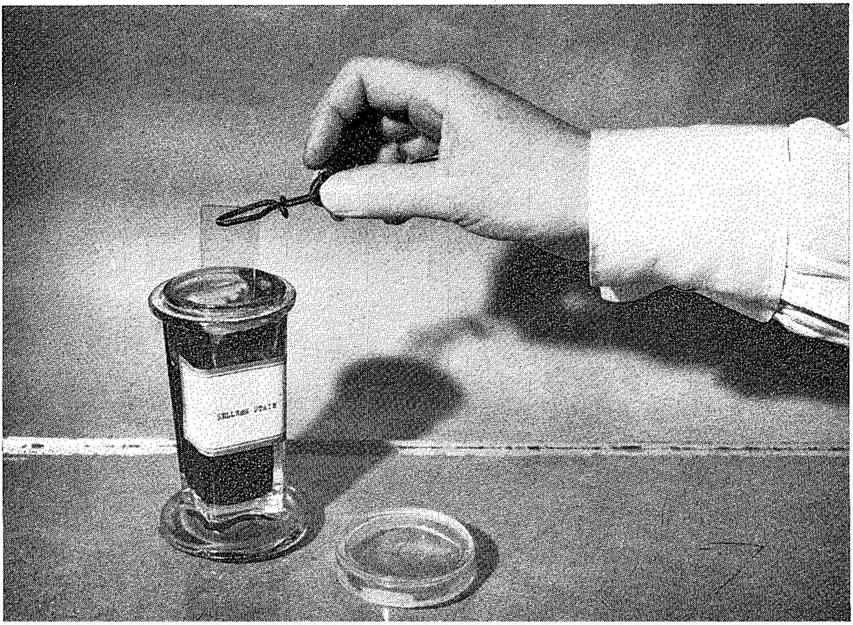
By courtesy of US DHEW-PHS-CDC

FIG. 7. CLOSE-UP OF SEVERAL IMPRESSIONS ON A SLIDE



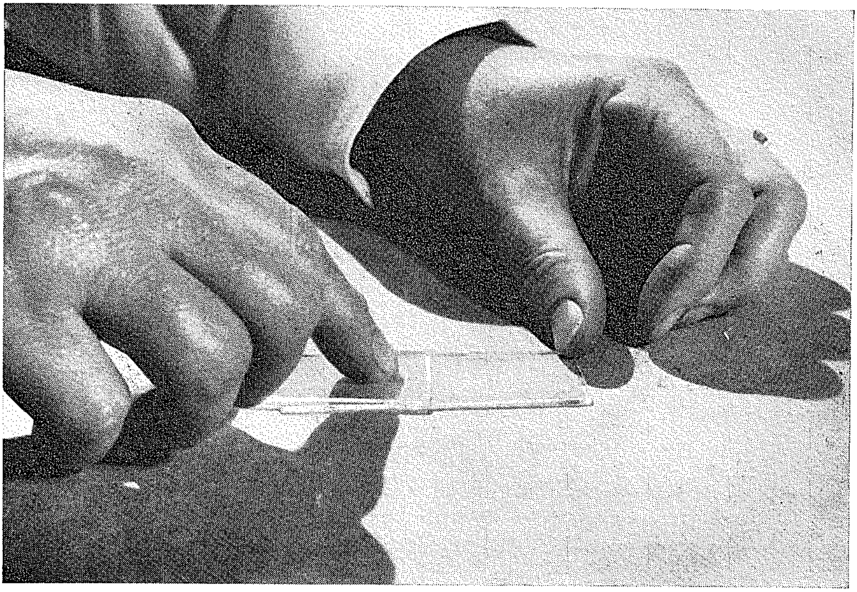
By courtesy of US DHEW-PHS-CDC

FIG. 8. IMMEDIATE IMMERSION OF SLIDE IN SELLERS STAIN WHILE TISSUE FILM IS STILL MOIST



thin film of tissue covering about three-quarters of the area of the slide.

FIG. 9. SMEAR METHOD OF SLIDE PREPARATION



By courtesy of US DHEW-PHS-CDC

In this spread-smear technique there is a copious concentration of tissue and a rather extensive area for examination. Care should be taken not to use too large a tissue-section, as this will result in an excessively thick film, making proper staining and microscopic examination impossible. The impression method, however, gives superior results.

" Rolling " method

The last method, the "rolling" technique, consists of cutting a piece of brain tissue about the size of a fresh garden-pea, and rolling or teasing it gently (cut surfaces downward) over the entire surface of the slide with a toothpick or wooden applicator.

The staining procedure of Sellers is recommended here because of its accuracy and simplicity. In this technique, no preliminary fixation is required, since the tissue film is fixed and stained simultaneously, making it one of the most rapid and easily handled methods.

The Negri Body: Differential Diagnosis

Although generally rounded in form, the Negri body may be found to assume any shape. At various times in different laboratories it has been demonstrated to be round, oval, spheroid, amoeboid, elongate, triangular, etc. By the same token, there is great variation in size; generally it is found within the limits of 0.24 μm and 27.0 μm. It is characteristically acidophilic in staining reaction, and takes on the pink to purplish pink colour in differential stains that use basic fuchsin or eosin with methylene blue as their base.

The position of the Negri body within the neurone is intracytoplasmic. Classically, it is found between the nucleus and one corner of the neurone, or in the prolongation of the cell body. However, it should be stressed here that the intracytoplasmic position of the Negri body can be expected with reasonable consistency only in histological sections of the brain. In the simple tissue-application techniques described above, the histological pattern is disturbed and one may very often see well-formed Negri bodies that appear to be entirely outside the neurone. Thus, in methods such as the impression, smear, or rolling techniques, the intracellular position of the Negri body is not required as a diagnostic criterion, and Negri bodies that satisfy the requisites of morphological identification, whether inside or outside the neurone, are sufficient to establish a positive diagnosis.

The most characteristic feature of the Negri body is its internal structure. It is this feature that serves as the essential criterion for positive identification in the techniques described in this section. The matrix of the Negri body has an acidophilic staining reaction, and contained within this magenta-red structure are small inner bodies (*Innerkörperchen*), basophilic granules that stain dark-blue to black. The size of these inner granules generally varies from 0.2 μm to 0.5 μm. Classically, the well-formed Negri body—the so-called textbook picture—will have its inner granules arranged in rosette fashion, with one large centrally placed body and a series of smaller granules arranged neatly around the periphery of the Negri body. It should be pointed out, however, that this picture is the exception rather than the rule, and it is very rare indeed that such an orderly arrangement of the inner granules is seen. For purposes of diagnosis it is sufficient to establish the presence of these dark-blue-staining granules, regardless of their numbers or pattern of distribution within the matrix of the Negri body.

There is universal agreement that the Negri body is specific for rabies, and its presence always indicates this infection. Furthermore, a fully formed Negri body cannot be confused with anything else. However, in the diagnostic laboratory other types of inclusion body are sometimes encountered in animal brains and, because of certain similarities, may be philic inclusion bodies of canine distemper or Rubarth's disease (canine infectious hepatitis, fox encephalitis) are occasionally encountered. These seem to occur more often in the thalamus and lentiform nuclei than in the hippocampus. By the same token, the brains of non-rabid cats and laboratory white mice occasionally contain non-specific acidophilic inclusion bodies when presented for rabies diagnosis. All these non-rabies inclusion bodies have the same staining characteristics for Sellers stain (see Annex, page 52), and they cannot be differentiated from each other with the techniques described above. However, the important thing is that these non-rabies inclusions, as a group, can be differentiated from Negri bodies with the use of Sellers stain. The following outline may be used as a guide in this differentiation :

Negri bodies	*Non-rabies inclusion bodies*
Presence of basophilic inner granules	Absence of internal structure *
Heterogeneous matrix	Homogeneous matrix
Less refractive	More refractive
Magenta (heliotrope) tinge	Colour more acidophilic (pinker)

* See Plate I D, facing page 72

Small atypical intracytoplasmic inclusion bodies are sometimes found in animals killed during the early stages of rabies. For that reason, it is imperative to hold biting and suspect dogs in quarantine, rather than to kill them immediately and send the brain to a laboratory for diagnosis (see page 29). There is a double reason for this. First, it will allow observation for symptoms of rabies, which may make possible a clinical diagnosis of the disease. Secondly, the longer the animal is allowed to live, the better the chance of obtaining a positive microscopic diagnosis. The length of clinical illness in rabies is directly related to the size, abundance, and development of Negri bodies. Thus, if the disease runs its full course, Negri bodies are likely to the more abundant and fully formed with good internal structure.

Biological Diagnostic Test : Preparation for Mouse Inoculation

Since Negri bodies cannot always be found in the brains of animals dying of rabies, it is important that animal inoculation for demonstration of the virus be done on Negri-negative specimens. Extensive surveys of large numbers of rabies cases have shown that 10–15% of cases proved positive by mouse inoculation had been missed by direct-smear microscopic examination for Negri bodies. It is therefore strongly recommended that laboratories that furnish rabies diagnostic services be equipped to carry out animal inoculation tests on Negri-negative brain tissues.

In the past, the guineapig and rabbit were considered the most suitable test animals for this purpose. Since the intracerebral injection of rabies virus into white mice has been shown to produce typical and constant infection, the white mouse is now considered the test animal of preference. The chief advantages of the mouse are the low cost, making it possible to use several animals for one specimen, the relatively short incubation period for street virus, and the consistency of production of Negri bodies in the brains of mice inoculated intracerebrally with street virus. Suckling mice are the most susceptible (see chapter 7).

A positive microscopic diagnosis is sufficient proof of the presence of rabies. When the microscopic examination proves Negri-negative or questionable, no time should be lost in taking samples of the cerebral cortex, cerebellum, and Ammon's horn on each side of the brain, plus a sample from the brain stem (medulla). These should be pooled in the emulsifier in preparation for the mouse inoculation test described in chapter 7, page 85. It is important to achieve completely representative sampling of all those parts mentioned on each side for pooling, since there is often great variability in the virus distribution through the brain.

Antibacterial Agents for Contaminated and Decomposed Specimens

It is often difficult to obtain from the field animal brains that are bacteriologically sterile. The head may have been in transit for a long time, or the animal may have been picked up long after death, or shot, or clubbed on the head. Also, the cause of death may have been a bacterial encephalitis.

Intracerebral injection of bacteria may cause the death of inoculated mice in one, two, three, or more days, before any rabies virus that may be present in the inoculum has had its full incubation period. On the other hand, inoculated mice may live long enough for rabies incubation to be complete, may pass through the typical rabies symptoms of tremors, paralysis, and prostration, followed by death, and may show typical Negri bodies and many bacteria in their brain smears.

When bacterial contamination is suspected—for example, if the animal brain is decomposed or when many bacteria are demonstrated on the original brain smears—it is best to treat the brain suspension with an antibacterial agent before inoculating it into mice. Of the following agents, penicillin and streptomycin will give the best results. If these antibiotics are not available, any of the other agents may be used.

(1) *Glycerol.* Place the brain specimen in pure glycerol for 48 hours.

(2) *0.5% phenol.* Make up 0.5% phenol in physiological salt solution. This is used as the diluent for making up the 10% tissue inoculum. Hold

temperature. If held for over 6 hours, keep the suspension overnight in the refrigerator.

(3) *1:5000 thiomersal.*[1] Make up a 1 : 5000 solution of thiomersal in physiological salt solution. This is used as the diluent for making up the 10% tissue inoculum. Hold for 6 hours or overnight. If held for 6 hours, keep the suspension at room temperature. If held for over 6 hours, keep the suspension overnight in the refrigerator.

(4) *Penicillin and streptomycin.* Add 500 IU of benzylpenicillin sodium and 2 mg (1560 IU) of streptomycin per ml of tissue suspension. Allow to stand for 30 minutes at room temperature before injection. This amount is usually enough, but for very heavily contaminated brains or salivary glands, as many as 1000 IU of penicillin and 3 mg (2340 IU) of streptomycin may be used.

In order to help detect the possible presence of contaminating bacteria, a portion of all tissue emulsions may be cultured in dextrose infusion broth or similar media, and streaked on a blood-agar plate. The recommended amount is about 0.1 ml of emulsion in 3 ml of broth. Incubate overnight at 37.5°C.

Early deaths (1–3 days) among the infected mice may be attributed to the presence of contaminating bacteria if the cultures show moderate to heavy growth and if many bacteria are found in the brain smears of the dead mice.

Annex

PREPARATION OF SELLERS STAIN *

Examination of slide

Time may be saved in microscopic examination by a study of the stained slide under low power at first, selecting areas containing numerous large neurones to be examined for Negri bodies under oil immersion (see Fig. 10 and 11).

Sellers stain shows the Negri body well-differentiated in magenta or heliotrope to bright red, with well-demonstrated dark-blue to black basophilic inner bodies. All parts of the nerve cell stain blue, and the interstitial tissue stains pink. Erythrocytes stain copper-colour (orange-tinged red) and can be easily differentiated from the magenta-tinged red of the Negri bodies.

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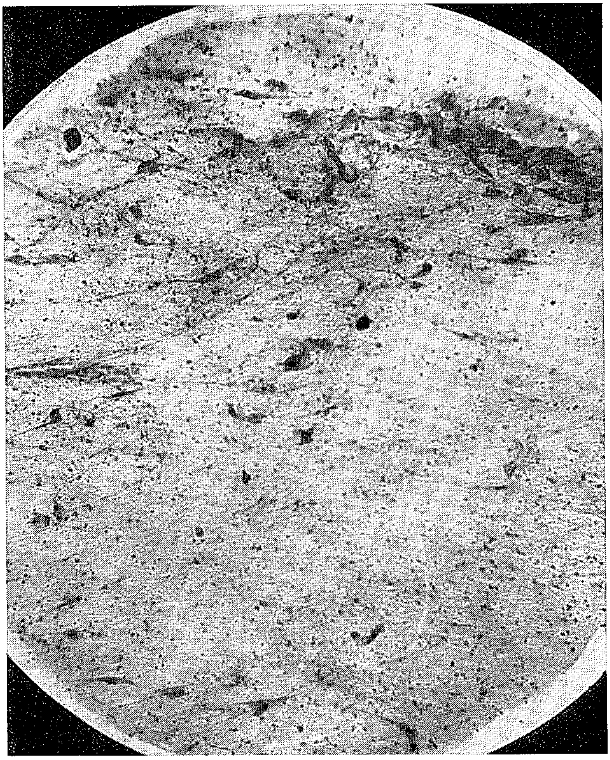
[1] Also known as thimerosal and as merthiolate.

* This annex was kindly contributed by Dr T. F. Sellers, Director Emeritus, Georgia Department of Public Health, Atlanta, Ga, USA.

FIG. 10. LOW-POWER VIEW OF IMPRESSION, SHOWING FIELD (UPPER HALF) RICH IN NEURONES FOR EXAMINATION UNDER HIGH POWER



By courtesy of US DHEW-PHS-CDC

Magnification ×200

Stock solution

(1) Methylene blue (Colour Index[1] No. 52 015, or Schultz[2] Index No. 1038)	10 g
Methanol (absolute acetone-free)	to make 1000 ml
(2) Basic fuchsin (Colour Index[1] No. 42 510, or Schultz No. 780)	5 g
Methanol (absolute acetone-free)	to make 500 ml

Other suitable dyes, such as those from Gruebler & Co., Leipzig, Gurd & Co., Ltd, London, and National Aniline Co., New York, may also be used.

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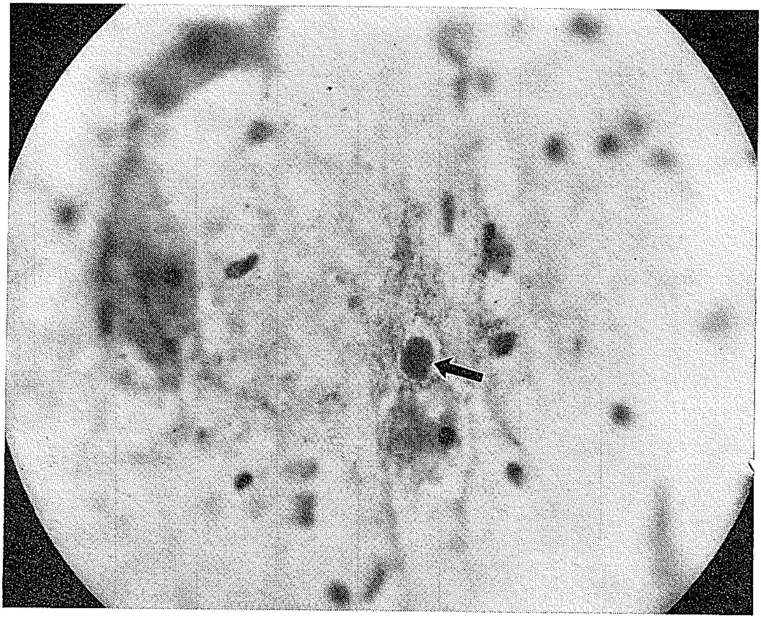
[1] Society of Dyers and Colourists and the American Association of Textile Chemists and Colorists (1956) *Colour Index*, 2nd ed., Bradford.



By courtesy of US DHEW-PHS-CDC

* Indicated by arrow

Magnification ×900

The stock solutions are stored in bottles with screw caps or ground glass stoppers. Certified biological stains are preferable. The dry dyes should be selected for high dye content—methylene blue, preferably not less than 85% ; basic fuchsin, preferably not less than 92%. While absolute acetone-free methanol is recommended, chemically pure (C.P.) methanol meeting American Chemical Society specifications may be substituted if desired.

Staining solution

Methylene blue (stock solution No. 1)	2 parts
Basic fuchsin (stock solution No. 2)	1 part

Mix thoroughly but do not filter. Store in container with a screw cap or ground glass stopper. The mixed stain improves after standing for 24 hours, and keeps indefinitely if protected from evaporation.

Adjustment of stain

When the stock staining solutions have been accurately prepared, the above proportions will usually produce a stain that will give the desired colour differentiation. However, it is as well to make a trial stain, and if the results obtained do not equal those illustrated in Plate I C (facing page 72) the stain may readily be adjusted. If the stroma is a bright red rather than rose-pink in the thinner areas, and the overall staining effect is reddish, the fuchsin is too dominant. Add methylene blue stock solution in measured amounts, checking with a trial stain after each addition until the desired colour balance is obtained. When the methylene blue is too dominant the Negri bodies are a deep muddy maroon colour and the nerve cells stain too deeply. Adjustment may be made with the fuchsin stock solution in this case.

If the stock solutions are protected from evaporation, more stain may be subsequently prepared using the stock solutions in the adjusted proportions.

Staining procedure

(1) Prepare smears or impressions in the usual manner (see page 42); no fixation is required.

(2) Immediately, *while the preparation is still moist,* immerse it in the staining solution for 1–5 seconds, depending on the thickness of the smear.

(3) Rinse quickly in running water, and air-dry without blotting.

In some regions tap-water is not satisfactory for rinsing purposes. The suitability of the water may be determined by comparing preparations rinsed with tap-water and others rinsed with distilled water containing 0.66 M phosphate buffer, pH 7.0.

When not in use, the stain must be kept in a tightly closed container to prevent evaporation, which tends to make the fuchsin too dominant. The addition of absolute methanol will restore the proper balance. It is convenient to keep the staining solution in a screw-capped Coplin jar for daily use. If this is not available the stain may be stored in a dropper bottle with ground glass stopper and the smear flooded with the stain. Staining in this manner will not be satisfactory unless the entire process can be completed within a few seconds.

The best results with the stain are obtained when the brain tissue is fresh. As decomposition sets in, the characteristic colour differentiation is affected, and although the Negri bodies retain their staining quality, the smear as a whole becomes too red, or at times too blue, and identification of the bodies becomes more difficult.

HISTOPATHOLOGICAL DIAGNOSIS

P. LÉPINE[1]

In principle, the histopathological diagnosis of rabies consists in recognition of the presence in the animal of acute encephalomyelitis which can be ascribed to a specific agent, namely the rabies virus.

If the animal has died from rabies, it is normally easy to detect the specific lesions ; if the animal has been killed, death may have occurred before the appearance of specific lesions (Negri bodies). Consequently, any animal in which the cerebrospinal axis (brain, medulla, ganglia) shows the slightest sign of lesions, particularly infiltrations, should be regarded as suspect, no matter how small the lesions may be.

After correct removal, the brain should be carefully dissected. Smears or impressions should first be examined for Negri bodies (see chapter 4, page 41). If any are found, this establishes the diagnosis. If this examination is negative, rabies is not thereby eliminated. A regular histopathological examination should be made of sections stained after embedding by a rapid method. At least 6 samples should be examined, corresponding respectively to Ammon's horn (both sides), the cerebral cortex (motor area), the cerebellum, the medulla, and a ganglion (gasserian or upper cervical).

The sections should be examined for :

(*a*) Signs of meningo-encephalomyelitis, i.e., meningitis, meningeal infiltration, perivascular cuffing, parenchymatous infiltration, formation of encephalitic nodules (Babès's nodules), and ganglion infiltration with satellitosis and neuronophagia (lesions of van Gehuchten and Nélis) (see Fig. 1 and 2).

These lesions may be detected by any staining method (e.g., haematoxylin-eosin, polychrome methylene blue). They show the existence of encephalomyelitis and enable a *tentative* diagnosis of rabies to be reached.

(*b*) Specific lesions. The different types of neurones should be examined for Negri bodies and lesions of fixed-virus rabies.

The Negri bodies are found especially in the central pyramidal layer of Ammon's horn (see Fig. 3) and the hippocampus, in the lower loop and the middle layer of the ganglioneurones of Ammon's horn and, less frequently,

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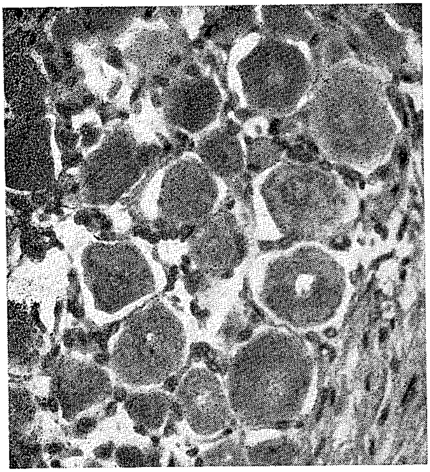
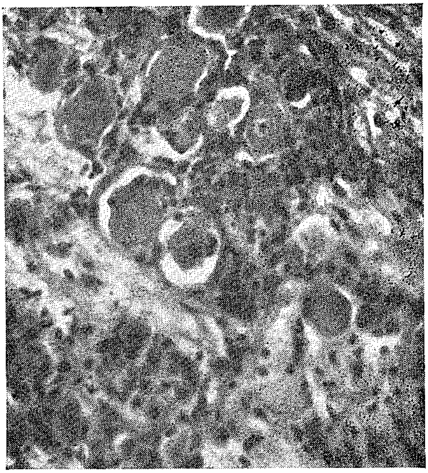
[1] Pasteur Institute, Paris, France.

FIG. 1. NORMAL GASSERIAN GANGLION



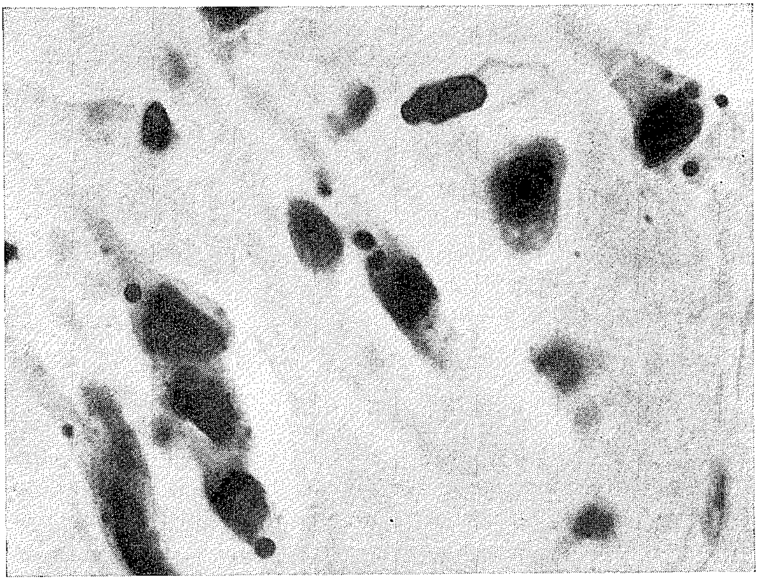
Ganglioneurones with a single layer of satellite cells

FIG. 2. RABIES : INFILTRATED GASSERIAN GANGLION



Infiltration, satellitosis, and neuronophagia (lesion of van Gehuchten & Nélis)

FIG. 3. DOG : STREET RABIES

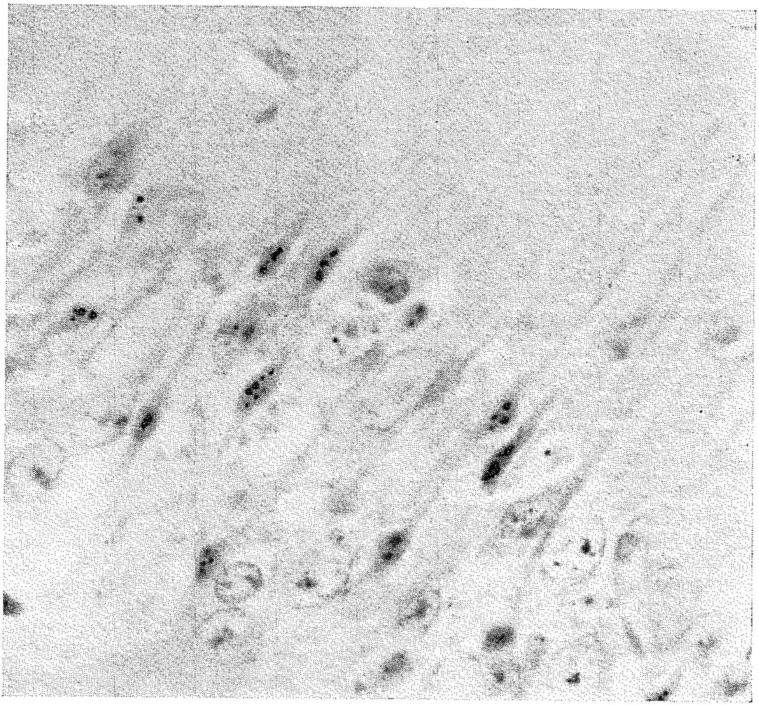


Mann stain

Magnification ×2000

Negri bodies in neurones of the central pyramidal layer of Ammon's horn the medullary nuclei. They may be present in very large numbers in the ganglia but are generally small in size.

FIG. 4. RABBIT : FIXED-VIRUS RABIES



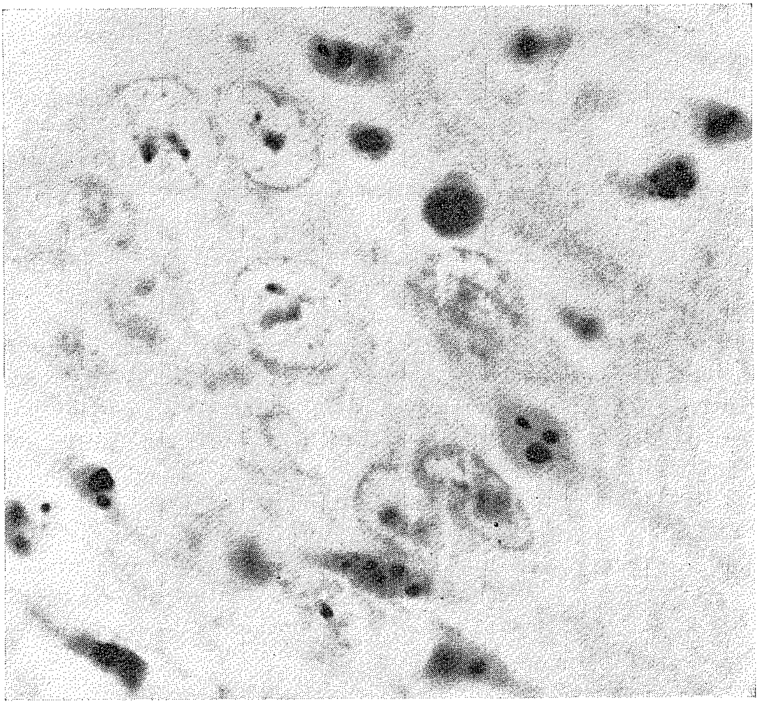
Lépine (fuchsin-safranine-blue) stain Magnification ×770

Polychromatic degeneration of neurones in outer layer of Ammon's horn

The lesions of fixed-virus rabies are found exclusively in the middle zone of the external layer of the cells of Ammon's horn (see Fig. 4 and 5). They always co-exist in a varying proportion with the lesions of street-virus rabies.

These lesions can be detected only by special staining methods (Mann, Sellers, etc., see Plate I, facing page 72). Their presence enables a *definite* diagnosis of rabies to the made.

FIG. 5. RABBIT : FIXED-VIRUS RABIES



Lépine (fuchsin-safranine-blue) stain Magnification ×1850

Detail of affected neurones : pyknosis of cellular bodies, with large quantities of polychromatic nuclear debris

BRAIN REMOVAL AND PREPARATION OF MATERIAL*

Large Animals (Dogs, Cattle)

The animal is firmly secured to the autopsy table or, better, the head is separated from the body and strapped to a wooden block hollowed out for the purpose. The operator and his assistants should wear thick rubber gloves to protect their hands. Make an incision on the midline of the skull through the skin, push aside the flaps of skin, and reflect the muscles and fascia as far as the base of the skull, proceeding from the crown to a horizontal line passing through the eyes. Saw transversely through the skull at eye level and make a symmetrically placed saw-cut through the occipital

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bone in prolongation of the cuts already made. Join up the saw-cuts by means of bone-cutting forceps, and lift up and push backwards the top of the skull. In the case of very large animals (large dogs, cows, etc.) a different method is preferable: make a longitudinal saw-cut on each side of the midline at about 1.5 cm from it, and join up these cuts by one or two transverse saw-cuts above the orbits and at the occiput so that the calvaria can be removed in two symmetrical pieces.

Once the calvaria has been removed, use fresh instruments to open the meninges, making use of serrated dissection forceps and a pair of fine, sterile scissors. The operation is performed by making an incision in the meninges, starting from the median region, along and on each side of the longitudinal sinus; next, a second incision is made perpendicular to the first and the meningeal flaps pushed upward and backwards. Again change instruments; cut through the medulla with a scalpel as low as possible and lift up the brain, proceeding from back to front and successively severing the pairs of cranial nerves. At the end of the operation the brain is toppled over forwards into a large sterile Petri dish where it rests on its upper surface. In hot weather, or if the brain is soft (cadaveric brain), it should be cooled to +5°C (refrigerator) to give it a firmer consistency before dissection.

Examination of the brain

Note whether or not there is congestion of the cerebral vessels or exudate in the meninges, etc. Dissect the brain as follows:

(1) With a brain knife separate the two hemispheres longitudinally, after having detached the cerebellum and the medulla.

(2) Look for the hippocampus and Ammon's horn. This may be done in two ways (see also chapter 4):

(*a*) Cut across the brain transversely, starting from the base behind the optic chiasma and proceeding towards the lower third of the convexity. The third ventricle appears on the cut surface; Ammon's horn is seen as a whitish fold resembling a large bean cut transversely, and can easily be removed.

(*b*) Alternatively, a longitudinal incision may be made externally in the posterior third of each cerebral hemisphere about 1.5 cm from the midline; the incision is continued through the grey matter and the white matter until a narrow groove, the third ventricle, is reached. The hippocampus will be seen on the floor of the ventricle in the form of a glistening white, semi-cylindrical bulge, extending laterally on each side (see chapter 4, Fig. 3, page 43).

Cut transverse sections 1–2 mm in thickness from each hippocampus. Take similar samples from the cerebral cortex (motor area), the cerebellum, and the medulla.

When using the impression method, at least 6 slides (2 for each hippocampus, 1 for the cerebral cortex, and 1 for the cerebellum) should be carefully inspected before deciding that the results are negative. If the results are negative, however, histological examinations are carried out.

Preparation of the tissue samples for histological examination

If the tissue is soft and difficult to section, prepare pieces of filter paper slightly larger than the tissue sample to be collected. Apply the piece of filter paper to the cut brain surface, grasp the edge of the filter paper with fine forceps held in the left hand, and with the right hand make a cut with a scalpel parallel to the filter paper and 2–3 mm from it, so as to remove the piece of brain, which is immediately submerged in the fixing agent together with the fragment of filter paper to which it is adhering.

Removal of material for inoculation

In operating, care should be taken to put on one side fragments aseptically removed from the same areas (cortex, hippocampus, cerebellum, medulla) for use in animal inoculation (see chapter 7, page 85). If the brain is received in good condition and can be assumed to be sterile, the fragments are removed before any examination is made. When the brain is infected, the fragments may be removed at any time and antibiotics added, as described in chapter 4, page 52.

Small Animals (Rabbit, Guineapig, Hamster, Mouse)

Rabbit

The animal, resting on its ventral surface, is attached to the autopsy tray, with the head at the edge of the tray. Using a serrated dissection forceps, the scalpel and the scissors, completely scalp the head from the nape to the muzzle, removing the ears and the upper eyelids. Moisten the exposed surface of the head with iodized ethanol and rapidly flame it with a bunsen burner. Holding the muzzle of the animal with a Farabeuf forceps in the left hand, open the brain pan with 3 cuts of the bone forceps. Make the first 2 in the front part of the head, from each orbit to the midline with an upward and outward movement of eversion, opening the brain pan (parietal and temporal bones) in 2 flaps, to the right and to the left. The third cut is made at the occiput, with a backward movement of eversion, which comfreed and the anterior part of the brain sectioned at the olfactory lobe; after severing the medulla behind the cerebellum, the brain is raised in order to cut the optic chiasma and is then placed in a Petri dish where it is dissected.

With the brain lying on its dorsal surface in the Petri dish, the ventral surface facing upwards, cut the cerebral trunk at the peduncle; then cut through the brain substance along a transverse slanting plane, starting from the optic chiasma and going towards the convexity, parallel to the posterior surface of the hemispheres and the cut surface of the cerebral trunk. A second cut made in the same way, parallel to the first and from 5 mm to 8 mm behind it, gives a transverse section of the brain that includes the hippocampal gyrus and Ammon's horn as well as the basal optic ganglion—areas of choice for the detection of Negri bodies—in addition to the cortical motor area (see Fig. 6 and 7). A transverse section of the cerebellum makes it possible to examine Purkinje's cells and the peduncular region. Finally, a slice cut from the end of the cerebral trunk gives a section of the medulla. To reach the gasserian ganglion, cut through the petrosal bone with the bone forceps at its insertion into the sella turcica; force apart the cut surfaces by everting the temporal bone downward and outwards. The gasserian ganglion is easily recognized from its whitish nodular appearance and its almost fibrous consistency. The ganglion is carefully removed, freed from fragments of bone, placed on a piece of filter paper, and immersed in the fixing agent with the other samples.

Guineapig, hamster

The animal is secured to the autopsy tray and the head thoroughly freed from skin. The skull is rapidly flamed. With the second pair of large sterile scissors, open the skull by means of 4 incisions encircling the skull cavity, the first one joining the two orbits and the other 3 made successively at the sides and the occipital bone. Remove the brain with the fine forceps and scissors and dissect it as described above for the rabbit. The gasserian

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Key to Fig. 6 (page 63)

A: Brain (mouse, rabbit): 1. cerebrum; 2. cerebellum; 3. medulla oblongata

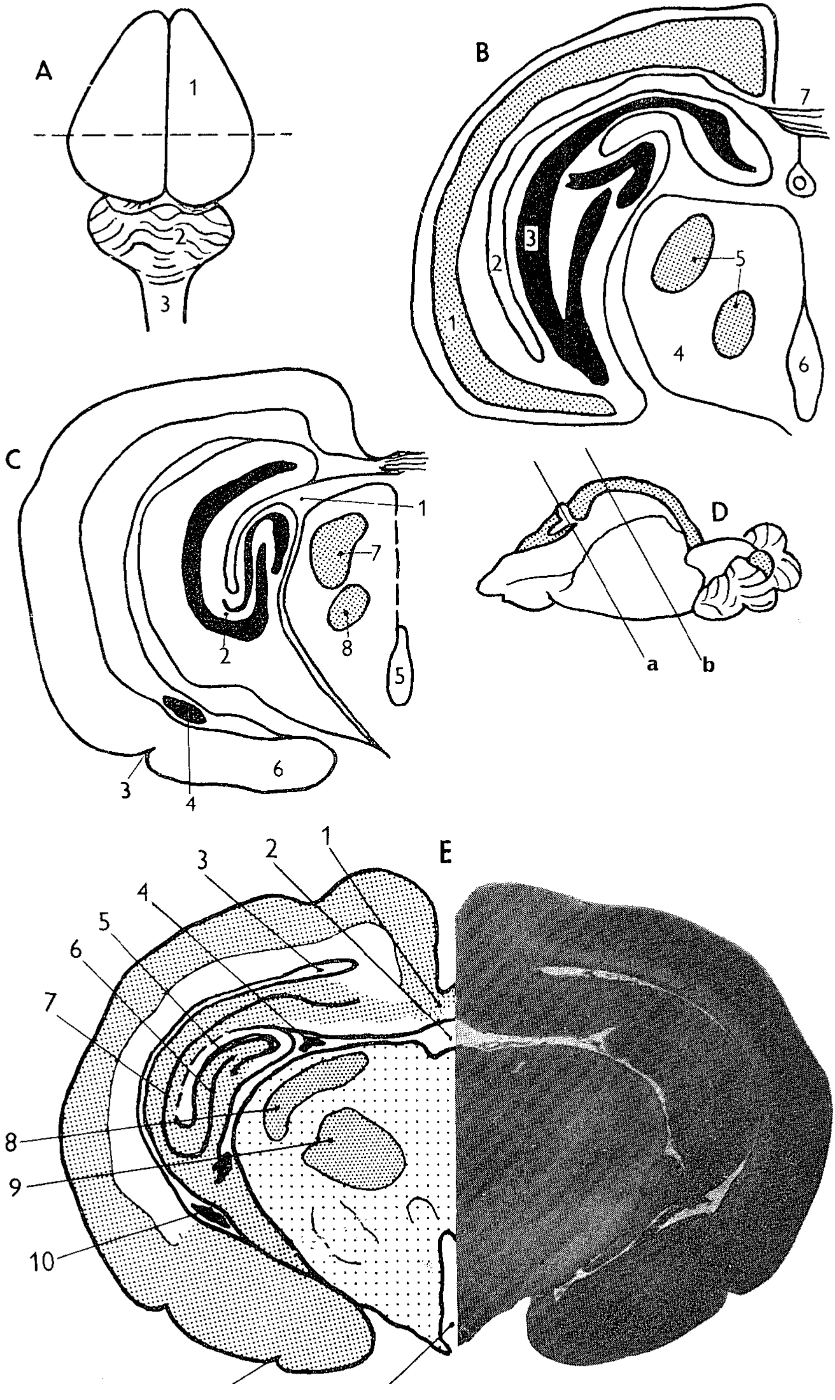
B: Section of normal mouse brain: 1. cerebral cortex; 2. lateral ventricle (choroid plexus); 3. Ammon's horn; 4. mesencephalon; 5. inner nuclei of grey matter; 6. median ventricle; 7. corpus callosum

C: Section of normal rabbit brain: 1. third ventricle; 2. Ammon's horn (middle layer); 3. rhinal fissure; 4. choroid plexus; 5. infundibulum; 6. hippocampal gyrus; 7. lateral geniculate ganglion; 8. ventral nucleus of thalamus

D: Rabbit brain after removal from cranial cavity: inverted, lateral aspect. Left—anterior portion; centre—temporal and parietal lobes; right—cerebellum. Section is performed anterior to *a* (through optic chiasma) and posterior to *b*; the portion *a-b* is immersed in fixative

E: Rabbit brain: transverse section *a-b*: 1. corpus callosum; 2. third ventricle; 3. lateral ventricle; 4. choroid plexus; 5. Ammon's horn (middle layer); 6. Ammon's horn (inner layer); 7. Ammon's horn (outer layer); 8. lateral geniculate ganglion; 9. ventral nucleus of thalamus; 10. lateral choroid plexus; 11. rhinal fissure; 12. infundibulum

FIG. 6. SECTIONS OF RABBIT AND MOUSE BRAIN



be found.

Mouse

The mouse is pinned, ventral surface downwards, on a sheet of cork, the limbs being spread out and fixed. First of all the 4 paws are fixed, the base of the tail is secured with a fifth pin, and a sixth is passed through the anterior extremity of the muzzle, which is pulled well forward. Remove the skin from the head. Treat the skull with iodized ethanol and flame it very gently with the pilot flame of the bunsen burner. Open the skull with the fine scissors, commencing by joining the orbits, then cutting laterally through the skull fairly low, and finally pushing back the flap thus obtained. Sever the medulla and the optic chiasma. Remove the whole of the brain and place it in a Petri dish. Separate the cerebellum. Cut through the brain transversely at the optic chiasma and along a plane parallel to the posterior surface of the brain, so as to obtain a cross section ; this is placed in the fixing agent, the anterior portion being reserved for smears and inoculations. Fix also a section of the cerebellum.

If examination of a ganglion is needed, the upper part of the spinal cord should be detached and removed together with the upper cervical ganglion.

EMBEDDING, STAINING, AND EXAMINATION FOR NEGRI BODIES

Rapid Mercury Sublimate Fixing Agent (Lépine & Sautter, 1936)

The following mixture is recommended for rapid fixation of the nervous system : equal volumes of glacial acetic acid, acetone, and saturated solution of mercuric chloride ($HgCl_2$) in absolute ethanol.

The mixture is made in advance and keeps perfectly well. Prepare the saturated solution of mercuric chloride in absolute ethanol in hermetically sealed bottles ; hasten saturation by keeping in the incubator at 37°C. The sections should be treated with Lugol's solution or iodized ethanol before staining, in order to remove the sublimate. Mixtures containing other components, particularly formaldehyde, give distinctly inferior results, as do mixtures to which water or hydrated ethanol has been added in order to slow down fixation. Tissues other than those of the nervous system are hardened by this fixing agent.

Rapid Fixation with Susa's Mixture

Rapid fixation can also be effected with Susa's mixture. For this purpose, the formula given by Langeron (1949) is recommended. First prepare a stock solution of the following composition :

sodium chloride	5 g
mercuric chloride ($HgCl_2$)	45 g
distilled water	800 ml

This mixture is stable and keeps indefinitely.

Immediately before use, prepare the following mixture :

stock solution	80 ml
acetic acid	4 ml
40% formaldehyde solution	20 ml
trichloroacetic acid	2 g

Cut the tissue in thin slices and fix for a few hours in this mixture. The fixation time should not exceed 48 hours. When fixation is complete, do not wash the tissue with water but treat it directly with iodized ethanol and then with 96% ethanol and absolute ethanol prior to embedding.

Fixation with Bouin-Dubosq-Brazil Mixture

When ultra-rapid fixation is not necessary and 24 hours are available for the purpose, the best fixing agent is beyond doubt Bouin-Dubosq-Brazil mixture. This can be used for all examinations not requiring a special fixing agent and is particularly suitable for the staining of nuclear and cytoplasmic inclusions. The formula is as follows :

commercial 40% formaldehyde solution	500 ml
96% ethanol	1100 ml
distilled water	100 ml
glacial acetic acid	120 ml
picric acid	8 g

Fixation is followed, without washing, by dehydration in 96 % ethanol and then in absolute ethanol. The specimens are embedded in paraffin, using the customary technique.

Rapid Method for Histological Embedding

For slices of brain tissue 1 mm in thickness, fixation is complete in 15–30 minutes if a rapid fixation method is employed. The tissue is transferred directly to absolute ethanol, with which it is treated for 20–30 minutes in two baths, followed by two changes of toluene and two of paraffin, each lasting 15 minutes. Including the time necessary for cutting and staining the sections, the preparation is ready for microscopic examination about 3½–4

It is sometimes advantageous to use a solvent for paraffin that is also miscible with water. The number of manipulations is reduced and for fairly thin tissue fragments there is a gain of time. The dioxan technique is suitable for this purpose.

Fix the tissue in thin slices, not exceeding 5 mm in thickness. In principle, any fixing agent can be used. It is preferable, however, to employ Bouin's dioxan mixture as given by Lison (1936):

dioxan saturated with picric acid	8.5 volumes
commercial 40% formaldehyde solution . .	1 volume
glacial acetic acid	0.5 volume

Next embed without washing, following the procedure given below (Mossman, 1937):

dioxan I	1 hour
dioxan II	1 hour
dioxan III	2 hours

followed by:

paraffin I	15 minutes
paraffin II	45 minutes
paraffin III	2 hours

Use fresh paraffin for embedding.

Take care to keep the dioxan in well-stoppered bottles (risk of chronic poisoning accompanied by anaemia caused by the vapour if the dioxan is kept in open bottles), and ensure that it remains anhydrous by adding pieces of quicklime (CaO).

Staining by Sellers's Method

Sellers's method may be used either in combination with rapid dioxan embedding (as described by Schleifstein, 1937) or for staining sections prepared in the normal way, adopting the technique of Nobel & Marton which ensures rapid and well differentiated staining. Thus, the simplicity of Sellers's method of staining is combined with the greater accuracy afforded by histological examination, so that the Negri bodies can be observed *in situ* in the cells, and all possibility of error is excluded.

The preparation of Sellers stain is described on page 52. After being freed from paraffin, the sections are stained by immersion in a mixture of 6 ml of basic fuchsin stock solution, 20 ml of methylene blue stock solution and 50 ml of absolute methanol.

The time required for staining depends on the thickness of the section; it is usually between 2 minutes and 10 minutes. The stained sections are washed in tap water, dried with filter paper without washing with alcohol, and mounted in balsam. The Negri bodies stain deep magenta red and appear as vacuoles in the blue-violet of the neurone. The internal structures of the Negri bodies are stained deep blue, as are the nucleoli of the nerve cells. The erythrocytes stain copper red, and it is impossible to confuse them with Negri bodies.

Staining of Negri bodies by Mann's Method

This classical method gives sections that are permanently stained, with very fine differentiation of the Negri bodies. It is an excellent demonstration method, but it requires time and a certain dexterity for full success.

Prepare the following mixture immediately before use :

methyl blue (*not* methylene blue), 1% aqueous solution	18 ml
1% aqueous eosin solution	23 ml
distilled water	49 ml

Stain for 24 hours at laboratory temperature, or for 6–14 hours at 38°C (in this case first treat the section with an ethanol-formaldehyde mixture to render the gelatine insoluble and prevent the sections from becoming detached).

Wash with tap-water and then rapidly with absolute ethanol.

For differentiation use the following alcoholic caustic soda solution :

1.5% solution of caustic soda in ethanol	1 ml
absolute ethanol	30 ml

Leave the section in this solution until it is stained pink (about 10 minutes). As soon as this stage is reached, wash the preparation well with tap-water. The section should take on a sky-blue colour ; if not, treat it with water containing acetic acid (2 drops of acetic acid in 40 ml of distilled water) for 1 minute.

Dehydrate rapidly (absolute ethanol) treat with xylene, and mount in balsam.

Result : Negri bodies, vermilion red ; nucleoli of the neurones, violet red ; chromatin, blue ; cells, dark blue ; stroma, pale blue ; erythrocytes, pink (see Plate I, A, B, facing page 72).

If phloxin B is substituted for eosin in the same proportion, the preparations obtained are less attractive (purplish-blue or mauve background instead of sky-blue) but the inclusions (Negri bodies) are more numerous

(Lépine, 1935)

After being well fixed, the tissue is embedded in paraffin, cut into thin sections, and freed from the paraffin. Stain for 10 minutes with the following mixture:

(1)	basic fuchsin	1 g
	50% ethanol	200 ml
(2)	0.2% aqueous solution of safranine.	

Mix equal parts of (1) and (2) and store in a dropping bottle; the mixture is fairly stable and keeps for some time.

Discard the stain, cover the section with a mixture of ethanol and acetone (equal parts) to remove excess stain, and wash rapidly: the section is coloured red. Stain for from 15 seconds to one minute with Unna's polychrome methylene blue (10% dilution) or with permanganate blue prepared by Stévenel's method (1918) and used undiluted. Discard the stain: the section is deep violet in colour. Differentiate in ethanol-acetone for a few seconds: the section becomes blue. Wash the preparation immediately in running water to remove the excess of blue stain, and again treat with ethanol-acetone. Then, without washing, partially dehydrate by shaking in a Borrel tube filled with absolute ethanol. The remaining stain is removed from the section which becomes differentiated, taking on a pink-lilac tint varying in paleness according to the thickness of the section. Rapidly complete dehydration in absolute ethanol, carefully remove the alcohol in several changes of xylene or toluene, and mount in balsam.

Result: stroma, very pale pink with nerve fibres a deeper pink; neuroglia and leukocytes, purplish-blue; neurones, light blue; chromatin, deep purple with the nucleolus a vivid red; pathological formations particularly well shown: nuclear inclusions and oxyphilic substances, bright pink; Negri bodies, poppy-red to mauve pink, with the internal structure lilac.

Method of Stovall & Black

A popular staining method used in the USA is that described by Stovall & Black (1940) using acetone fixation and sequence staining. The following description is taken from Lillie (1948).

1. Stain for 2 minutes in a 1% alcoholic solution of ethyl eosin (Colour Index[1] No. 45 386) adjusted to pH 3.0 with N/10 hydrochloric acid.
2. Rinse in water.

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[1] Society of Dyers and Colourists and the American Association of Textile Chemists and Colorists (1956) *Colour Index*, 2nd ed., Bradford.

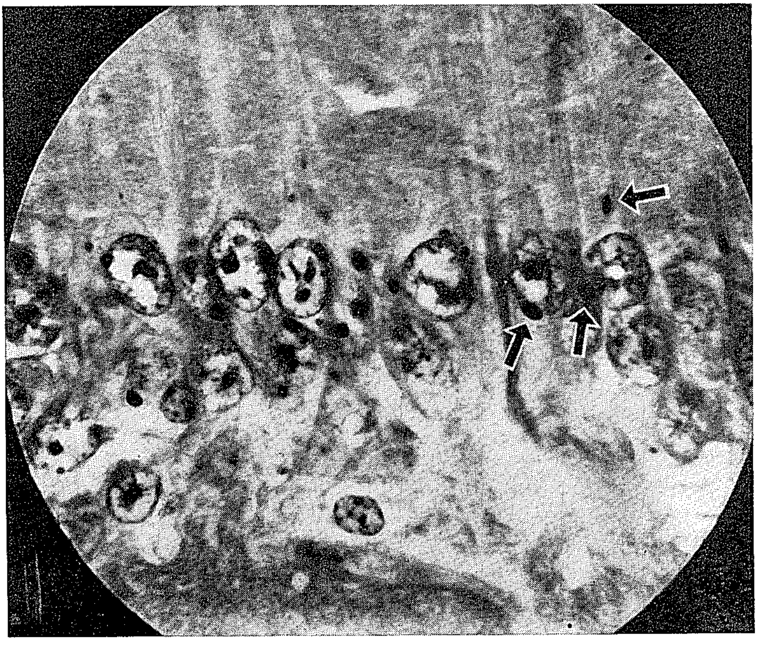
3. Stain for 30 seconds in 0.23% methylene blue in 22% alcohol buffered to pH 5.5 with acetic acid/sodium acetate buffer.
4. Differentiate in 0.38% acetic acid (13 drops in 60 ml of water) until sections are brownish red.
5. Wash, dehydrate, and clarify (xylene or toluene).
6. Mount in balsam.

Results : Negri bodies are brownish to pure red, nucleoli pale blue, other structures pink.

Lillie also recommends the following modification of the above method (Mossman, 1937), which has given good results (see Plate IE, facing page 72) :

1. To 90 ml of absolute ethanol or 94 ml of 95.5% ethanol add 3.25 ml of 1% acetic acid and make up to 100 ml with water ; dissolve 950 mg of ethyl eosin in this mixture. Stain for 2 minutes in this solution.
2. Wash in alcohol and in water.
3. Counterstain in 0.5% methylene blue if the section was fixed in formaldehyde, or in alum-haematoxylin if the section was fixed in ethanol.
4. Differentiate in 0.25% acetic acid for 2–5 minutes.
5. Wash, dehydrate, and clarify.
6. Mount in balsam.

FIG. 7. STREET RABIES : AMMON'S HORN (MIDDLE LAYER)



Mann stain Magnification ×1100

(Levaditi et al., 1948)

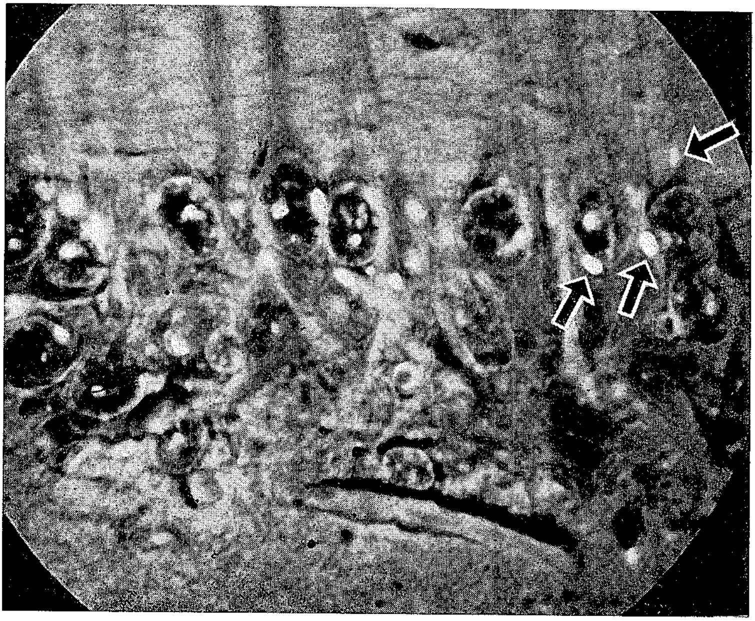
This method makes use of the fluorescence of the Negri bodies when they have been impregnated with a fluorochrome and excited by exposure to light of a wavelength of 400 nm (4000 Å).

(1) Fix the brain tissue and embed in paraffin as usual. Make fine sections, mount on slides, and treat successively with xylene, absolute ethanol, and water to remove the paraffin.

(2) Next, stain by immersion for 30 minutes in an aqueous 0.2% solution of thioflavine S. Without washing the preparation, treat it for a few seconds with absolute ethanol and then with toluene. Mount in balsam between the slide and the cover-slip, applying as thin a layer of balsam as possible. It should first be ascertained that the balsam does not contain substances that fluoresce in ultraviolet light.

(3) Next examine with the fluorescence microscope, using a filter to block wavelengths longer than 515 nm (5150 Å). Locate Ammon's horn under

FIG. 8. STREET RABIES : AREA OF BRAIN, TISSUE BLOCK, AND CELLS IDENTICAL WITH THOSE OF FIG. 7



Thioflavine S

Magnification ×1200

Appearance under fluorescent light; the Negri bodies, stained with thioflavine S, are strongly fluorescent and stand out clearly against the dark background of the preparation.

low magnification and examine with high magnification, using glycerol or liquid paraffin as immersion liquid.

The Negri bodies are at once conspicuous by their vivid fluorescence (see Fig. 7 and 8) and brilliant azure-blue colour. The background of the preparation is pale-yellow, the cellular cytoplasm brilliant yellow, and the nucleoli and erythrocytes pale blue.

It should be noted that this fluorescence method, which takes advantage of the affinity of the Negri bodies for fluorescent stains, is quite different in principle from immunofluorescence methods (see chapter 6, p. 73), where the staining is brought about by specific fixation on the infected cells of antibodies coupled with the fluorescent substance.

Other Staining Methods

Among the innumerable methods that have been recommended for staining Negri bodies the following also give good results :

(1) Lentz's method (1907) : this is a variation of Mann's method.

(2) Gallego's method (1925), using acid ferric chloride as mordant, followed by staining by Ziehl's method and with picro-carmine.

(3) Romanowsky-Giemsa method, using a buffered Giemsa stain and the technique of Lillie & Pasternack (1936).

(4) Schleifstein's method (1937) : a combination of Sellers's method with rapid dioxan embedding (see also page 66).

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DESCRIPTION OF PLATE I *

A. Street rabies in dog brain. Many typical Negri bodies situated in the cytoplasm and in the prolongations of the neurones. Some contain haloes and "Innerkörperchen". (Mann stain : × 1550)

B. Fixed-virus rabies in tissue culture. Intracytoplasmic inclusions of different shapes and sizes. (Mann stain : × 1550)

C. Street rabies in bovine brain. An inclusion in the cytoplasm of a degenerated neurone (to the right of the nucleus) is a typical Negri body with basophilic "Innerkörperchen". (Sellers stain : × 1550)

D. Cat brain section with non-specific inclusions in the cytoplasm. No "Innerkörperchen". (Mann stain : × 1550)

E. Street virus in monkey ganglia. Invasion of gasserian ganglion in the inflammatory and neuronophagic process ; chromatolysis and Negri bodies. (Haematoxylin-eosin : × 660)

F. Fixed virus in tissue culture. Cytoplasmic inclusion in fixed rabies demonstrated by the specific immunoperoxidase reaction[1] (× 1550).

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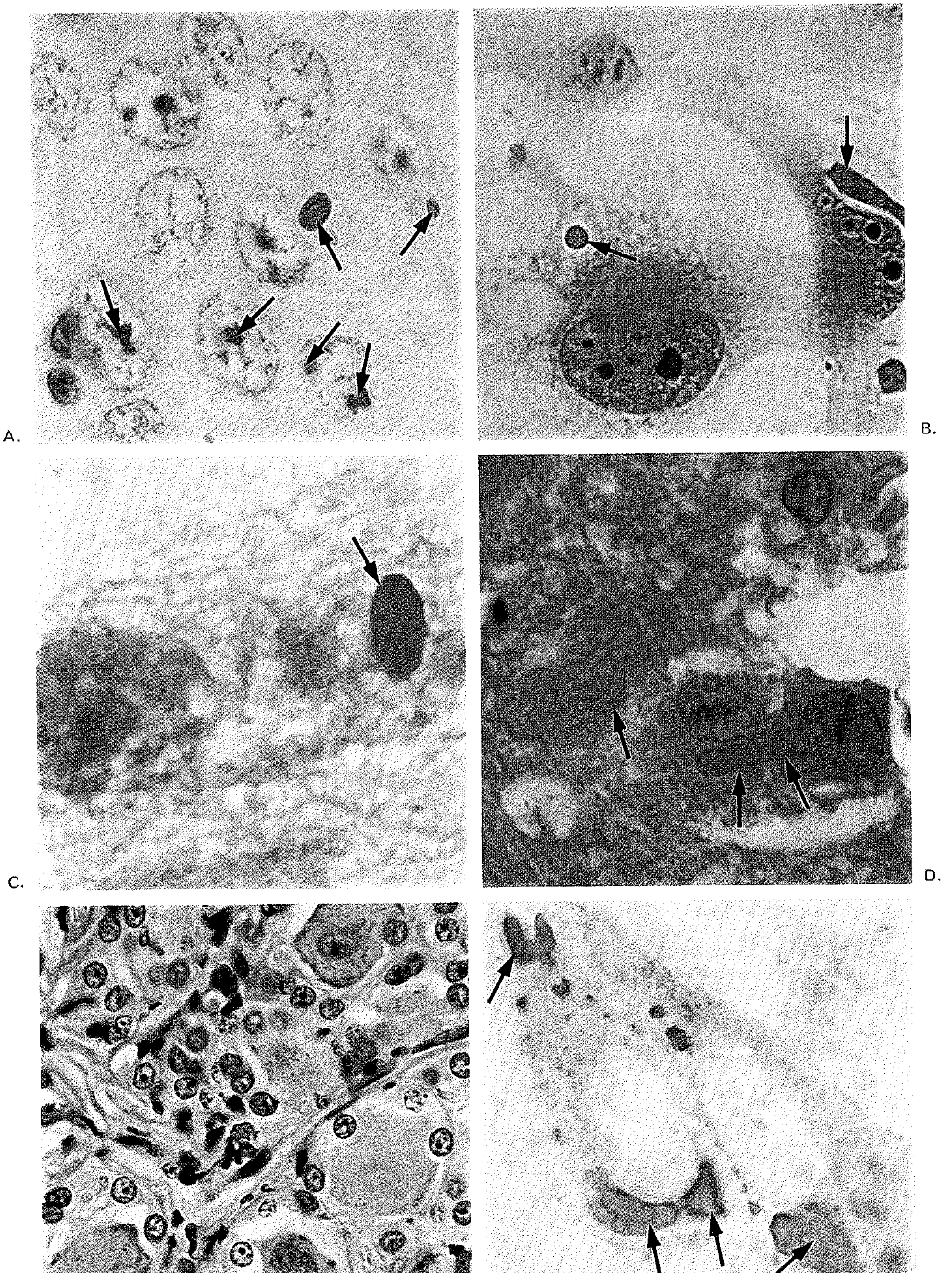
* Colour photographs kindly supplied by P. Atanasiu and J. Sisman, Pasteur Institute, Paris, France.

[1] See Appendix 6, page 358.

PLATE I



CHAPTER 6

THE FLUORESCENT ANTIBODY TEST

D. J. DEAN[1] *& M. K. ABELSETH*[2]

The fluorescent antibody (FA) test is the most accurate microscopic test at present available for the diagnosis of rabies and should be employed by all laboratories undertaking such work. Considerable experience, more or less constant practice, and satisfactory reagents and equipment are required. In competent hands the test is fast, comparatively inexpensive, and more accurate than either the examination of films or sections by recommended procedures or mouse inoculation tests. Fresh, frozen, or glycerolated material may be examined. Diagnosis can be made accurately in most instances in minutes or hours, whereas the examination of sections or animal inoculation requires days or weeks.

Fundamental Principles

Although fluorescence microscopy is not new, its use to detect reactions between an antigen and its antibody stems from the work of Coons & Kaplan (1950); their techniques were subsequently adopted for use in rabies diagnosis by Goldwasser & Kissling (1958). The procedure consists in labelling antibody with a fluorochrome, allowing the labelled antibody to react with specific antigen if present, and observing the product of the reaction under the fluorescence microscope. A substance is said to fluoresce if, upon absorbing light energy at a certain wavelength, it emits light of another wavelength.

Antigens reacting with antibodies tagged with fluorescein isothiocyanate, the dye most frequently used with rabies, appear under ultraviolet light as brightly coloured, apple-green or greenish-yellow objects against a dark background, which may or may not contain nonspecific fluorescing material. The character and intensity of the colour may be modified by the use of filters. Fluorescein isothiocyanate is available commercially in powdered form and may be stored for long periods without deterioration.

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[1] Director, Division of Laboratories and Research, New York State Department of Health, Albany, USA.

[2] Director, Laboratories for Veterinary Science, Division of Laboratories and Research

satisfactory quality equipped with a dark-field condenser and an ultraviolet light source.[1] Since only a small portion of the incident radiant energy is converted to fluorescent light, examinations should be made in a darkened room employing the most intense source of light available ; both excitation and barrier filters are used. Although a monocular head will yield more light, a good quality binocular microscope will furnish sufficient light and is preferred in diagnostic laboratories where a large number of specimens are being processed. Light used for excitation is of shorter wavelength than that emitted by the preparation. Blues and greens are excited only by ultraviolet light, while yellow and red fluorescence may also be excited by blue-violet light.

Several manufacturers make complete outfits acceptable for fluorescence microscopy and units are available for converting standard microscopes of satisfactory quality for use in fluorescence work. Guidance in the selection of a microscope should be obtained from those experienced in fluorescence microscopy and the manufacturer should be required to demonstrate its capabilities prior to purchase. Apochromatic or fluorite lenses allow higher apertures and therefore present brighter images. Achromatic lenses can be used but provide less brilliance. The use of a dry substage dark-field condenser should be considered since it eliminates the use of oil between the slide and front lens of the regular condenser. Large amounts of oil on the microscope may be a hazard since the methods described to process rabies specimens do not inactivate the virus.

Methods and Procedures

Conjugate

Once good microscopic equipment has been obtained, the quality of fluorescence is primarily dependent upon the quality of the conjugate used. Some laboratories use sera from hamsters immunized according to schedules similar to those developed by the California State Department of Health.[2] Other laboratories use equine or goat antisera for conjugation. Since commercial conjugates of acceptable quality are now available, only laboratories familiar with such procedures should attempt to make their own. Such conjugate should be checked and standardized with one of known quality before it is used for diagnosis. Conjugates prepared in the laboratory should be lyophilized to prevent loss of potency. Good quality conjugates should have a minimum of nonspecific fluorescence at low dilutions and should be capable of being diluted as much as 1 : 20 or more to guard against this problem. After a lot of conjugate has been prepared, dilutions should be made and tested to determine which one is most satisfactory for routine diagnosis.

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[1] For general reviews of fluorescence microscopy, see Price & Schwartz (1956), Kawamura, jr (1969), and Goldman (1968).

[2] See annex, p. 80, for detailed method of preparation.

As needed, lyophilized conjugate is removed from the deep freeze and reconstituted to its original volume with sterile distilled water. It should then be filtered to remove extraneous materials. The desired amount of reconstituted conjugate is added to the appropriate volume of normal or infective mouse-brain suspension prepared with egg yolk as later described. For example, if satisfactory staining is obtained with a final dilution of conjugate of 1:40, 0.1 ml of conjugate is added to 3.9 ml of mouse-brain suspension. The remaining undiluted conjugate is stored at 4°C until used. After storage, the conjugate may need to be centrifuged lightly to remove precipitate before addition to the mouse-brain suspension. Diluted conjugate may be used for a period of a week or so, provided precautions are taken to safeguard against undetected decay of the product or contamination. If conjugate is prepared as outlined in the annex, the final dilution may be made with sterile distilled water rather than with the normal mouse-brain suspension as described. This is possible since very little nonspecific fluorescence is found in conjugates prepared in this manner. One should be aware that conjugate prepared in the laboratory may contain nonrabies fluorescein-labelled antibodies if multiple antigens are present in the antigen used in the preparation of antisera. Such conjugates may react when autogenous tissues are being examined. Care must be exercised therefore in the selection of pure rabies antigen.

Preparation of slides

Two methods of preparing slides are commonly used. They are those described for examination for Negri bodies in chapter 4. Uniform, thin films can be prepared by grinding hippocampal or other brain material without alundum to a uniform paste with a sterile mortar and pestle or by cutting this material into small pieces with scissors. Films provide a greater number of cells for examination. This method is preferred in preparing brain material that has deteriorated. Impression smears of such material would be too thick for successful examination.

Control slides

Control films or smears are usually prepared from the entire brains of mice or young hamsters inoculated intracerebrally with rabies street virus and sacrified when moribund. After fixation in acetone, slides can be stored in a dry condition at −20°C for long periods before use ; however, When infected mouse brain is used as antigen for preparing antisera, it is usually necessary to dilute conjugates with a 20% normal mouse-brain diluent. The diluent is a 10% suspension in phosphate-buffered saline (pH 7.4) of the yolk of eggs embryonated for 6–7 days. After centrifugation for 10 minutes at 1000 *g*, the supernatant is dispensed in glass containers in aliquots sufficient for 1–7 days' work and stored at −20°C until used. Suspensions of infected mouse brain are similarly prepared using brains from young adult mice inoculated intracerebrally with a 1 : 100 to 1 : 1000 suspension of the CVS strain of fixed-rabies virus and harvested when moribund.

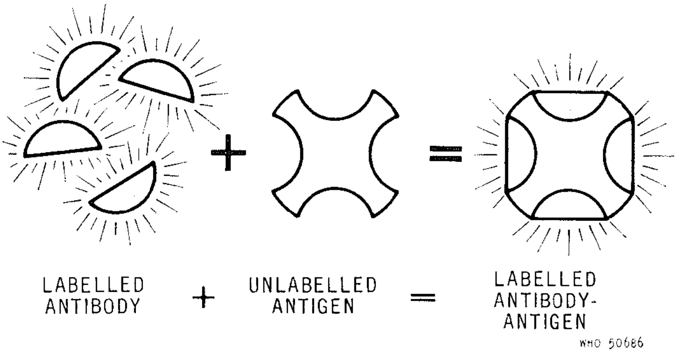
Immunofluorescent staining

Many variations have been developed in the procedure for immunofluorescent staining; however, the direct method is the most satisfactory and practical for rabies diagnosis and will be the only one described.

The direct test

The direct method, depicted schematically below, consists in applying a suspension of rabies antibody, previously labelled with fluorescein isothiocyanate, directly to films, impression smears, or sections of tissue under examination.

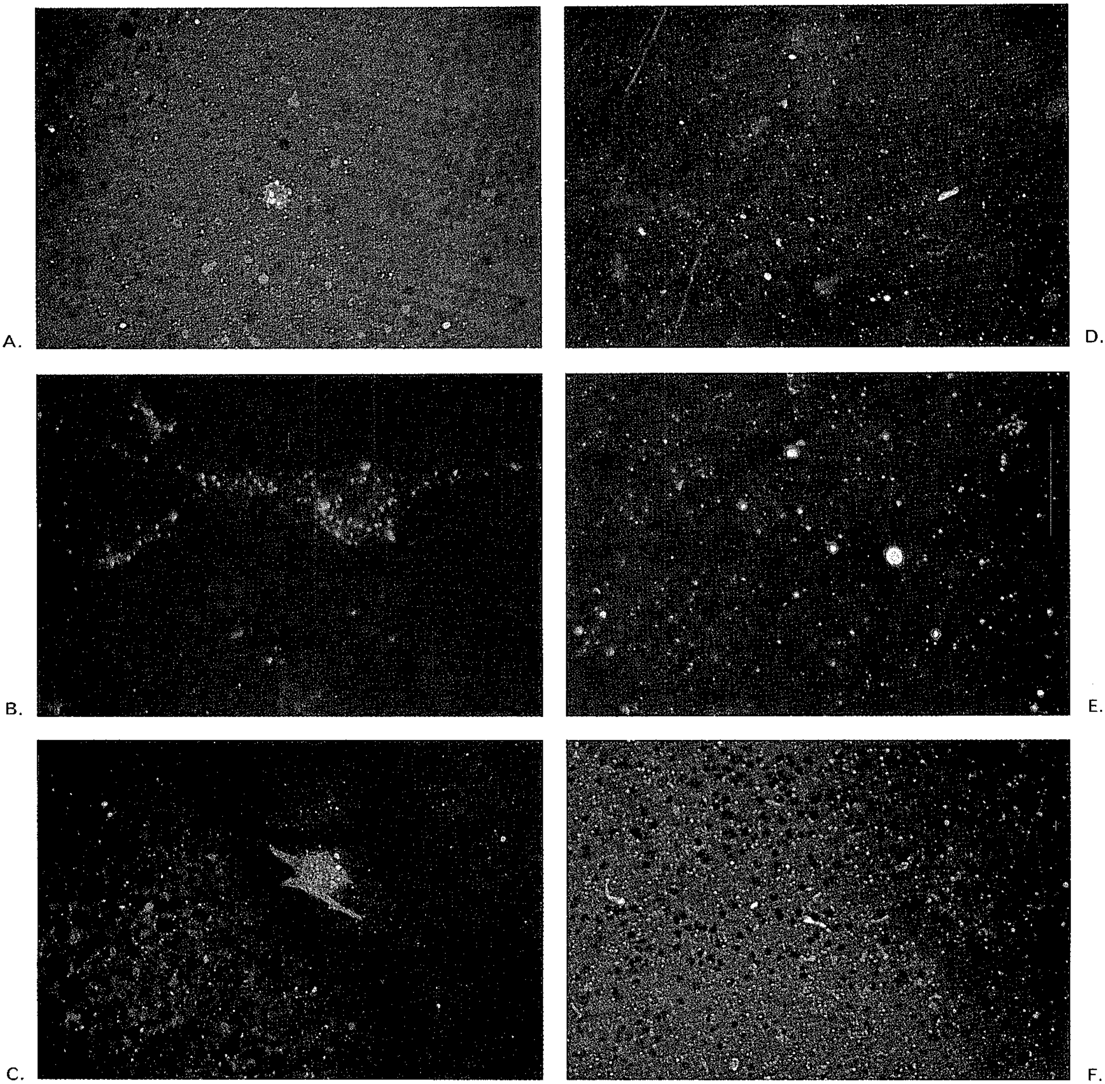
DIRECT METHOD OF DETECTING RABIES ANTIGEN



Schematic representation of reaction of unlabelled rabies virus (antigen) with fluorescein-tagged antibody to yield fluorescein-labelled antigen-antibody product.

Not less than 4 films of salivary gland and/or central nervous system material per specimen should be examined before reporting field specimens as being fluorescent-negative; they should include 2 made from hippo-

PLATE II



Photomicrographs of brain material treated with hamster conjugate diluted 1:30.

A. Cerebellum — cow. Note fine particles (dust) as well as antigen in nerve cell.
B. Neurone — dog.
C. Neurone — cow.
D. Hippocampus — fox. Typical variation in size. Suitable as positive control slide.
E. Ammon's horn — cat.
F. CVS — mouse.

campus and 2 from a paste prepared by grinding in a mortar, without diluent or alundum, equal portions of material from the hippocampus, the cerebellum, and brain stem from the region of the pons. Component parts of the brain may be examined separately if desired. In examining bovine specimens, the cerebellum and stem are tissues of choice. When the mouse test is used, the remaining ground tissue is diluted with distilled water containing 5% of normal horse serum to obtain a 10% tissue suspension and then injected intracerebrally. A direct comparison of the results of fluorescence and mouse inoculation is thus obtained.

When specimens are received in 50% glycerol-saline (preservative), it is imperative that the tissue be washed several times in saline. Glycerol may interfere with the test because acetone combines with glycerol to mask fluorescence. In such cases good results may be obtained by omitting the acetone fixation step.

Films are air dried, placed in a Coplin jar or other suitable container, covered with cold acetone, and held in a freezer at −15°C to −20°C for 2–4 hours; excellent results are also obtained with overnight fixation. After fixation, slides are removed from the acetone and air dried. If films are used, 2 suitable uniformly thin areas 2.5 cm long are demarcated on the slide with a wax marking pencil. Impression smears, 2 per slide, are similarly demarcated. Slides, including previously prepared controls, are next placed on glass rods strategically arranged across the top of a small sterilizing tray or other suitable container.

One area of film or one impression smear is stained by placing 2 drops of the diluted conjugate within the area marked by the wax pencil. The other film or smear is stained in the same way with conjugate similarly diluted with infected mouse-brain suspension. Conjugate should be spread uniformly, without disturbing the film, either by rotating the slides or with an applicator stick or toothpick. A fresh stick should be used for each area stained. The slides are placed on a rack in a container (a shallow boiling pan with suspended glass rods similar to that used for bacterial staining is suitable). Water is added to the pan, which is then covered and placed in an incubator at 37°C for half an hour. This procedure creates sufficient humidity to prevent drying of the test material.

After incubation the slides are washed by dipping in phosphate-buffered saline, pH 7.4, then further rinsed by immersing for two successive 10-minute periods. Coplin jars are convenient for this purpose. The slides are then removed and air dried in a vertical position. When dry, one drop of 50% buffered glycerol (pH 7.6) is added and cover glasses are mounted over the areas to be examined. When properly stained, the positive control film and unknown films containing rabies antigen will contain brilliantly fluorescing apple-green or greenish-yellow structures, varying in size from tiny bodies, which are commonly called sand or dust and are barely visible, tions from both the central nervous system and the salivary glands. Control slides, prepared from known infected brain material and stained with conjugate diluted as described earlier, should *always* be examined before and after scanning the test films to ensure that the equipment is operating satisfactorily and that films are properly stained. When large numbers of slides are examined consecutively, control slides should also be interspersed with the unknown slides, e.g., every tenth slide.

No fluorescence should be seen on films or smears prepared from conjugate diluted with infected mouse-brain suspension as described above; this control procedure is therefore important in determining the specificity of the fluorescence and minimizing the number of false positives.

Accurate methods of confirming rabies in man or animals prior to death are not at present available. A test has recently been reported (Schneider, 1969) in which impressions are made of the cornea of the living suspect and examined by the FA test. This method, called the cornea test, has not been completely evaluated but should be considered where diagnosis of a clinical case is necessary, keeping in mind that a negative result may not rule out rabies.

Discussion

The FA test for detection of rabies antigen has been perfected to such an extent that it is the quickest, most reliable method available, both for diagnostic and research purposes. Errors can usually be traced to faulty equipment, unsatisfactory conjugate, lack of control slides, and lack of experience in reading slides.

Fluorescent-positive, mouse-negative specimens can occasionally be expected, since the FA test detects inactivated as well as live antigen. In this respect, fluorescence tests are more sensitive than tests in mice. All test slides submitted to 50 state health department laboratories in the USA in 1968 were diagnosed correctly. Some laboratories report, on occasion, mouse-positive, fluorescent-negative specimens, but this incidence is declining with increased competence in the performance of the FA test.

From 1966–70, nearly 15 000 specimens were examined in our laboratory. Of these, 802 of a total of 804 were found positive by the FA test. In only 2 cases was the mouse test positive and the FA test negative. Insufficient sampling was responsible for these results. Since samples found positive in the FA test are not routinely injected into mice, data are not available on the reverse situation. It is perhaps significant that 2 major state laboratories, New York and California, reporting almost perfect correlation, utilize conjugate of hamster origin that can be diluted as much as 1 : 50 prior to use, thus minimizing or eliminating the problem of nonspecific fluorescence. It is advisable to test in mice all fluorescent-negative specimens involving human exposure in order to maintain a constant check on the FA test. It should be kept in mind that suckling mice (1–3 days of age) are more susceptible than weanling mice and therefore should be used where rapid results are important. Since antigen can be detected by the FA test as early as 3 days after inoculation, one mouse per day can be sacrificed beginning on day 3.

Errors may also result from the use of unsatisfactory or inadequately adjusted equipment, nonspecific fluorescence, gradual and unrecognized loss of light from a deteriorating bulb, failure to use adequate controls, and colour blindness on the part of the operator. One should guard against tissue from a positive slide becoming attached to one that is negative.

Adequate sampling is an important factor. Since rabies virus is usually transmitted from the site of inoculation to the central nervous system via the peripheral nerves (Dean et al., 1963), we routinely examine brain-stem material either separately or as part of the composite sample. On repeated occasions, the brain stem has been positive when films prepared from the hippocampus, cerebellum, or cerebral cortex were negative. Brain-stem sampling is particularly advantageous when animals are killed early in the course of the disease.

With satisfactory conjugate, counterstains such as rhodamine-conjugated albumin are rarely needed. When rhodamine is used in dilutions of less than 1 : 80, loss of specific fluorescence occurs in direct proportion to the concentration of rhodamine used. Dilutions of 1 : 80 or greater are apparently sufficient to stain background material a light brick red leaving the greenish-yellow rabies antigen in sharp contrast. There is no apparent correlation between the condition of the specimen examined and the degree of autofluorescence. Good to excellent fluorescence with little or no autofluorescence has been observed in badly decomposed specimens and, occasionally, is encountered in fresh specimens. With experience, however, one is able to differentiate between specific and nonspecific fluorescence without great difficulty. The importance of using inhibited conjugate (diluted as described earlier) cannot be overemphasized since this procedure will rule out nonspecific fluorescence.

Examination of the salivary glands should be made routinely in the case of bats involved in human exposure and may yield valuable information with other species as well. Salivary glands are more difficult to sample adequately and to process than brain material. Since grinding with mortar and pestle is not usually satisfactory owing to the character of the tissue involved, films are customarily prepared from the cut surface or surfaces of the gland by the impression smear technique and stained as with brain material used for the direct microscopic test.

The fluorescence seen in salivary gland preparations is more diffuse than that seen in brain tissue. Autofluorescence, varying from blue to red, is examination can provide accurate information in the diagnosis of rabies in bats.

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Annex

PRODUCTION AND LABELLING OF ANTIRABIES SERUM

I. PREPARATION OF RABIES IMMUNE SERUM FOR FLUORESCENCE MICROSCOPY *

Host animal : Hamster

Tissue source of immunizing antigen

1. Inoculate 0.05 ml of a 1% suspension of stock CVS rabies virus (containing 500 units each of penicillin and streptomycin per ml) intracerebrally into a group (about 30) of 4-week-old hamsters.
2. Harvest infected brain and spinal cord tissues when hamsters become ataxic, paralysed, or moribund (usually 5–6 days).

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* Method as used by the California State Department of Health, with some modifications.

3. Store as whole tissue at −20°C until suspension can be prepared.
4. Grind brain and spinal cord tissue to a 20% suspension in 0.85% NaCl solution.
5. Transfer tissue suspension to 40-ml centrifuge tubes and centrifuge at 600 *g* for 15 minutes in a refrigerated centrifuge, if possible.
6. Remove supernatant fluids from tubes and pool into a flask.
7. Distribute pooled fluids into 20-ml ampoules in 10-ml amounts.
8. Label, flame seal, and shell-freeze each ampoule and store at −70°C.

Inactivation of antigen with β-propiolactone

1. *Reagents*
   (*a*) β-propiolactone[1]
   Store at −20°C.

   (*b*) Bicarbonate-saline

   Add 1.68 g of $NaHCO_3$ and 0.1 ml of a 1% stock solution of phenol red indicator to 100 ml of 0.85% NaCl solution. This solution need not be sterile if freshly prepared.

   (*c*) Disodium hydrogen phosphate

   Dissolve 14.19 g of Na_2HPO_2 in 100 ml of distilled water. (The solution may have to be heated gently to get the salt into solution.) Distribute the solution in 2.0-ml amounts in 12-mm × 75-mm tubes and sterilize it in an autoclave.

   (*d*) Sterile distilled water

2. *Inactivation procedure*
   (*a*) Hold all reagents and materials (except the β-propiolactone and disodium hydrogen phosphate) at 4°C for several hours prior to use.

   (*b*) Remove five 10-ml ampoules of the 20% suspension of hamster CVS rabies virus from the freezer (−70°C), thaw the contents in lukewarm water, and transfer to cold 50-ml graduated cylinders.

   (*c*) Obtain an ampoule of β-propiolactone (3.0 ml) from storage at −20°C and open in a cold room.

   (*d*) Remove the contents of the ampoule with a cold 5.0-ml syringe with a 20-gauge needle and transfer to a 12-mm × 75-mm tube.

   (*e*) Place 10 ml of cold distilled water in a cold 25-ml volumetric flask with a cold 10-ml pipette.

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pipette, fill the flask to the 25-ml mark with cold distilled water. This gives a 10% solution of β-propiolactone.

(*g*) Shake the flask vigorously until a homogeneous suspension is obtained. Occasionally a small amount of the β-propiolactone fails to go into suspension but this does not seem to affect the results. However, if a fairly homogeneous suspension does not result, this may indicate that the β-propiolactone has deteriorated and fresh material should be obtained.

(*h*) Measure 48 ml of cold bicarbonate-saline into a cold 250-ml volumetric flask.

(*i*) Prepare a 0.4% β-propiolactone solution by adding 2.0 ml of the 10% β-propiolactone solution to the volumetric flask.

(*j*) Add 50 ml of cold 20% hamster CVS rabies virus to the flask. This gives a final concentration of 0.2% β-propiolactone in the virus preparation.

(*k*) Shake the flask vigorously so as to mix the contents thoroughly.

(*l*) Place the flask in a water bath at 37°C for 2 hours.

(*m*) Remove the flask from the water bath and note the pH (phenol red indicator). Adjust the pH to 7.2–7.4 if necessary with a few drops of disodium hydrogen phosphate solution.

(*n*) Distribute the β-propiolactone-inactivated virus into rubber-stoppered ampoules in 10-ml amounts and store at −20°C.

Preparation of immunizing antigen

1. Remove the appropriate amount of β-propiolactone-inactivated hamster rabies virus (allow a slight excess of 1.0 ml per animal to be immunized) from the material stored at −20°C and thaw in a lukewarm water bath.
2. Prepare the adjuvant mixture of 9 parts of white mineral oil (viscosity 75–85 at 100°F)[1] and 1 part of Arlacel A.[2]
3. Add the virus suspension by increments to the adjuvant, shaking after each addition until equal volumes of each are combined.
4. Shake the completed mixture for 5–10 minutes in a shaker until homogeneous.

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[1] A suitable grade, white mineral oil No. 3, is manufactured by the Standard Oil Co., New Jersey, USA.

[2] Manufactured by Atlas Chemical Industries, Inc., Wilmington, Del., USA.

Inoculation schedule

1. Give a series of 4 weekly injections of 2.0 ml of immunizing antigen intraperitoneally.
2. Give one injection of 1.0 ml of 5% active hamster CVS rabies virus intraperitoneally during the 5th week.
3. Give one injection of 1.0 ml of 10% active hamster CVS rabies virus during the 7th week.
4. Bleed animals 10–14 days after the last injection.
5. Separate the serum and store at −20°C until conjugation procedures can be carried out.

II. LABELLING OF RABIES IMMUNE SERUM

1. Centrifuge the serum at 4 500 *g* for 60 minutes at 4°C. Decant the supernatant through a double acid-washed filter paper[1] pre-rinsed in 0.85% NaCl solution. Collect the filtrate and record the total volume. Pack the serum in ice and slowly add an equal volume of cold saturated ammonium sulfate solution, pH 7.2. Allow to stand overnight at 4°C.
2. Centrifuge the precipitated globulin at 800 *g* for 30 minutes at 4°C and resuspend the precipitate in phosphate-buffered saline (PBS), pH 7.4, 0.14 M, equal to one-half the original serum volume. Check the total volume and add an equal volume of cold saturated ammonium sulfate solution. Allow to stand for at least 60 minutes. Centrifuge the suspension at 4 500 *g* for 30 minutes at 4°C. Resuspend the precipitate in PBS, equal to one-half the original volume of serum.
3. Dialyse the globulin against PBS for 2–4 days, with magnetic stirring. Add 5.0 g of Dowex A62-X4 anion exchanger for each litre of PBS. The dialysis bath should be changed twice daily. Check the dialysate with saturated barium chloride for the presence of the sulfate ion. The last two baths should be PBS without the anion exchanger.
4. Collect the globulin and record the total volume. Determine the total protein by the biuret reaction. Add carbonate-bicarbonate buffer, pH 9, equal to one-tenth the total volume, and then add 0.03 mg of fluorescein isothiocyanate per mg of protein. Allow this to combine overnight at 5°C with stirring.
5. Dialyse the tagged globulin against PBS with Dowex anion exchanger at 5°C for 2–4 days, changing the bath twice daily. The dialysis bath can be checked for the presence of fluorescein with an ultraviolet lamp.

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fuge the conjugate at 4 500 *g* for one hour, then dispense in 1-ml aliquots. The conjugate may be stored frozen at −20°C or lyophilized.

6. The optimum dilution of the conjugated antiserum must be determined by serial dilution. A sample ampoule of the conjugate should be titrated in twofold serial dilutions and used to stain positive control slides. The last dilution that produces excellent immunofluorescence should be considered the FA titre of the conjugate. Before use, reconstitute the frozen or lyophilized conjugate to the dilution of optimum fluorescence with physiological saline containing thiomersal[1] 1 : 10 000, buffered to pH 7.6. Filter once through a Millipore filter, HAWP 0.45 µm. This preparation can be stored for at least 2 weeks at 4°C. If after 2 weeks or longer nonspecific fluorescence becomes a problem, the conjugate should be centrifuged at 4 500 *g* for 30 minutes at 4°C.

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[1] Also known as thimerosal and merthiolate.

CHAPTER 7

THE MOUSE INOCULATION TEST

H. KOPROWSKI[1]

The mouse inoculation test, in spite of its simplicity, depends greatly on the accuracy of its performance for dependable results.

Choice of Mice

Strain

White mice of any breeding strain may be considered generally suitable, although preference should be given to the Swiss albino strain since it is very susceptible to rabies virus and it is easy to maintain the breeding stock in the laboratory. If the Swiss albino stock is not available, however, almost any breed of mice, except grey wild mice, can be used, because a genetically resistant strain has not yet been found. Grey wild mice should be excluded, not because of insusceptibility but because of the difficulty in restraining these animals in cages during the observation period.

Age

Mice of all ages are susceptible to intracerebrally introduced rabies virus, but suckling mice up to 3 days of age are more sensitive. The use of adequate numbers of suckling mice has permitted individual virus isolations that would have been missed if only young adults had been inoculated. If available in sufficient numbers, suckling mice should be used routinely in addition to other diagnostic tests. Since confusion may arise from non-specific deaths, the combined use of the mouse inoculation test with suckling mice and the FA test is recommended.

Sex

Mice of both sexes are equally susceptible to rabies virus. It is inadvisable to keep older mice of the same sex in one cage since they are apt to kill each other in fights before the observation period is completed. This applies especially to males.

General health

It is imperative that the animals chosen for inoculation be in good health. It is important to know the history of the breeding colony, and it is advisable to inspect the animals closely before inoculation.

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diarrhoea, should disqualify the animals immediately. If the mice are sent to the laboratory from a considerable distance, it is advisable to postpone inoculation for a minimum period of 3 days in order to let them rest and become adjusted to changed conditions. In such cases it may be equally important to leave a few animals uninoculated in order to observe the death-rate among "normal" mice as compared with inoculated animals.

Preparation of Supposedly Infectious Material for Inoculation

Choice of tissue

Either the brain or the salivary-gland tissue of a suspectedly rabid animal may be used for virus isolation. Detection of the virus is more frequently possible in the brain than in the salivary gland. However, from the epidemiological and epizootiological points of view, it is important to examine the salivary glands for the presence of virus.

Although it is relatively immaterial which part of the brain tissue is chosen for the preparation of the suspension, preference may be given to Ammon's horn, the cerebellum, and parts of the cortex. When salivary-gland tissue is chosen, the submaxillary glands are those most likely to show the presence of rabies virus. Also, when salivary-gland tissue is used, it is always advisable to mince it before grinding.

Grinder

The choice of grinder depends to a certain degree on the amount of tissue available. If more than 3–4 g of material are available, a small Waring Blendor should be preferred over any other type of grinder. If the amount of tissue is less than 3 g (which is more usually the case), or if a Waring Blendor is not available, the following grinders or grinding devices may be substituted, in the order of preference listed.

(*a*) *TenBroeck grinder* : This grinder is a handy tool, easy to assemble, manipulate, clean, and sterilize, but only brain tissue may be used in it since salivary-gland tissue is too tough for it to grind properly. A slight disadvantage is its fragility. If improperly used, it may break while being manipulated ; and the grinder may be easily damaged during cleaning, sterilizing, etc. In regard to personal safety, the Waring Blendor and the TenBroeck grinder, if properly used, have some advantage over the other grinding devices.

(*b*) *Mortar and pestle* : This is a time-honoured method of grinding and it has the advantage that, with the help of an abrasive (e.g., sterile sand), even the toughest tissue can be properly ground up. The mortar and pestle cannot be operated under such sterile conditions as can the Waring Blendor or the TenBroeck grinder; it can, however, be easily cleaned and sterilized, and stands wear and tear for a long period of time.

Diluent

The choice of diluent may be left to the user but an isotonic salt solution is preferable, although mice have been known to withstand easily an intracerebral inoculation of sterile distilled water. The following diluents may be considered in order of their availability:

(*a*) *A physiological salt solution containing varying amounts of animal serum (10–50% concentration)*: This is by far the commonest diluent used. It should be carefully ascertained, however, that the donor animal has never been vaccinated against rabies. It is therefore advisable to avoid the use of dog, cat, or cattle serum. Normal sheep serum seems to possess some "antiviral" properties that are absent from rabbit serum. The rabbit should therefore be considered the preferred source of blood. It is preferable to inactivate the serum for 30 minutes at 56°C before using it as a component of the diluent. Diluent-containing serum can be sterilized only by filtration through bacteria-retaining filters.

(*b*) *Other diluents*:

(i) skim milk;
(ii) bovine serum albumin in buffered salt solution;
(iii) physiological salt solution in distilled water.

These are not particularly recommended, although rabies virus seems to be relatively non-susceptible to the inactivating action of salt solution, in contrast to such viruses as eastern or western equine encephalomyelitis.

Note: If a tissue suspension is to be frozen and stored, a 50% solution of serum in water should be the preferred diluent.

Bacterial sterility

There is no need to add antibiotics to suspensions of *brain tissue* if the material has been handled with reasonable precautions at the autopsy and dispatched in a sterile container. A 50% glycerol solution is highly recommended for preserving the material since, in addition to its preserving qualities, it exerts a strong bacteriostatic action (see chapter 4, page 51). If there is any doubt, however, it may be safer to add enough streptomycin and penicillin to the suspension to obtain a concentration of 1560 IU of streptomycin and 500 IU of penicillin per millilitre of the final suspension. If antibiotics are added, it is best to let the suspension stand for at least *glands*. Regardless of the addition of antibiotics, the salivary-gland tissue suspension should always be cultured for possible bacterial contamination. Beef-infusion broth, thioglycolate medium, and blood-agar are considered to be good culture media for this purpose. If bacterial growth is observed, an attempt should be made to identify the bacterial agent. If the results of the mouse inoculation test are equivocal (see page 92) it may be advisable to test the pathogenicity for mice of the bacterial contaminant by intracerebral inoculation.

Concentration of infected tissue in the suspension

This is optional. If the suspension is for storage, it is advisable to prepare a 20% suspension by weight. The weight of the tissue in grams multiplied by 4 gives the required amount of diluent in millilitres. However, if the suspension is to be used for inoculating mice intracerebrally, a 10% suspension by weight should be given preference. Either dilute the 20% suspension by adding an equal volume of diluent, or prepare the suspension by multiplying the weight of tissue in grams by 9 in order to obtain the required amount of diluent in millilitres.

In attempting to isolate certain strains of rabies street virus, the "self-sterilizing neuro-infection" phenomenon may be suspected on very rare occasions, and in such cases it is advisable to dilute the tissue suspension more than 10% (see page 92).

Centrifugation and filtration

If the equipment is available, it is advisable to centrifuge the tissue suspension for 5 minutes at 150–200 *g* to remove the gross particles. However, if no centrifuge is available, it is perfectly feasible to inject mice intracerebrally with a 10% uncentrifuged brain suspension. Salivary-gland suspensions, if uncentrifuged, must be filtered through one or two layers of sterile gauze in order to prevent the death of the animals from trauma.

Inoculation of Mice

Choice of syringe

Syringes that can measure accurately 0.03 ml (single mouse dose) should be used. Thus $^1/_4$-ml tuberculin syringes should be considered first, followed by ½-ml or 1-ml tuberculin syringes. For intracerebral inoculation, 27- or 26-gauge (0.40–0.45-mm) needles 1–1.5 cm long should be selected. Larger-gauge needles cause trauma to the brain substance.

Anaesthesia

It is strongly recommended that mice be anaesthetized before they are inoculated : inhalation of ether is the best form. A battery jar with a specially fitted wire bottom may be used for the purpose. If no such device is available, pentobarbital sodium injection should be considered. It is a good procedure to have the work-table used for mouse inoculation drawn well away from the wall, so that an assistant can etherize the mice from a position opposite the operator.

Inoculation technique

There is a wide choice of methods of inoculation. For a right-handed operator the following technique has been found satisfactory.

Anaesthetized mice are laid on the left side, the legs pointing towards the inoculator. With the thumb of his left hand, the inoculator supports the lower jaw of the animal, with his index finger behind the skull of the animal. Pressure should be very slight in order to avoid the asphyxiation and death of the animal. In his right hand, the inoculator holds the syringe in a horizontal position parallel to the table surface and perpendicular to the head of the mouse, with the needle pointing towards the inoculator. With a quick thrust, the needle is pushed through the skull of the animal at the place that can best be described as the apex of an imaginary angle, the arms of which point to the animal's right eye and right ear. The needle easily penetrates the bone and should then be further inserted for about 0.1–0.2 cm into the brain tissue. When the inoculator uses a 1.5-cm needle, he should be careful not to penetrate too far since he may then end by giving the injection into the base of the skull. The plunger is pushed to the next 0.03-ml mark and the needle is then gently withdrawn. For intracerebral inoculation, move the inoculated mice away from the syringe hand (i.e., right-handed persons pass the inoculated mice to the left). This is to prevent crossing hands, which may result in catching a finger on the needle held in the opposite hand.

The inoculated mice are immediately placed in a can or box previously prepared and identified by a tag bearing the mouse-group number or any other particular identification mark. If a large series of inoculations is made, it is worth while checking the number of living mice in each series after the entire experiment is finished. If any animals are found dead, an equal number of new animals should be inoculated and added.

Under no conditions should the same syringe be used for inoculation of two different suspensions. If an adequate number of sterile syringes is not available, each syringe should be boiled between inoculations, and care should be taken to let it cool before filling it with inoculum.

It is well always to use the same rigid precautions to establish good in the operator, or in the animals with which he is working, if the disease can be transmitted by such exposure. Virus may be spread from table to hands, and if these are not washed properly it is possible to contaminate subsequent specimens during grinding of tissue in a mortar—for example, virus may fall from the hands or sleeves into the mortar. Mice laid on the table after etherization may awake and have to be put back in the ether jar, thus contaminating it. Should other studies be carried out using the same ether jar, virus may be deposited on the head of normal mice and be carried into the brain by the inoculation procedure. The work-table must be considered contaminated until washed with soap and water. Dilute bichloride of mercury is a good disinfectant for table tops. Cresol and phenol solutions are no better than water for the most common virus contaminant of mouse tissue, namely, the mouse encephalomyelitis virus, and several other viruses ; phenol has no appreciable virucidal action on rabies virus.

When inoculation of all the mice is finished, the syringe and needle may be rinsed in water, provided this is done gently with the point of the needle well below the surface of the water in the sterilization tray. The inoculation of mice and harvesting of mouse tissues is best done on trays that can be sterilized and covered with absorbent paper backed with polyethylene or on a sheet of aluminium foil covered with a sheet of absorbent paper. Instruments used for individual specimens must be sterilized by boiling, by high-temperature dry sterilization, or by autoclaving ; sterilization by flaming between taking speciments may not invariably kill the virus.

Observation of Inoculated Mice

Although rabies virus will only rarely cause signs of illness in mice before the fifth day after intracerebral inoculation, it is advisable to check mice daily, beginning with the first post-inoculation day. The number of mice found normal, sick, or dead is recorded on a mouse-history card which remains on file as a permanent record of the experiment. The observation period should extend for a minimum of 21 days after inoculation. Only rarely will rabies virus be detected in the inoculum later than 21 days after the inoculation.

The following signs may be noted :

(*a*) Ruffled fur ; (*b*) tremors when held in the air by the tail with a pair of forceps ; (*c*) lack of coordination of hind legs—note gait when placed on table and made to move ; (*d*) paralysis ; (*e*) prostration (near death). Letter symbols denoting the different signs are recorded each day on the mouse-history card.

MOUSE-HISTORY CARD

No. Date Virus

Strain Passage Preparation

Volume Dilution

DATE																					
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1																						
2																						
3																						
4																						
5																						
6																						
Checked by																						

Age of Mice Route

LPR 542

Deaths occurring 24–48 hours after intracerebral inoculation are attributable to causes other than rabies virus (trauma, bacterial contamination, other viruses). For diagnostic purposes, 1 or 2 mice may be sacrificed each day, beginning on the fifth day, and a search made for Negri bodies as well as for the presence of rabies antigen as demonstrated by immunofluorescent antibody staining. Frequently, an early diagnosis is thus obtained, particularly in instances where certain strains of street virus might take between 1 and 3 weeks to kill the mice.

Note : Clinical signs of illness in inoculated mice cannot be considered characteristic of rabies. Although signs of paralysis 5 days or more after inoculation may give grounds for suspecting the presence of rabies virus, the same signs may be observed in numerous other viral, bacterial, and protozoal infections that involve the central nervous system of the mouse. Definite evidence of the identity of the virus is obtained with the virus neutralization index test (see chapter 8, page 94).

Further Passages of Infected Material

If it is desired, brain tissue from mice that have succumbed to infection after inoculation with the original virus may be made into a suspension as described above; it can then be stored, used in neutralization tests, or inoculated into another group of mice.

Removal of Mouse Brain

The brains of all mice that have died during the night, as well as the brains of those mice that have been sacrificed when prostrate, should be symptoms of prostration (terminal symptoms of rabies) should be killed with chloroform.

The dead mouse should be pinned to a dissecting board with the dorsal aspect of the body facing upward. Only two pins are necessary, one through the nose and one through the base of the tail. Three pins or spring clips may also be used : one through each of the forelegs, and one through the back of the tail.

After disinfection with ethanol, the skin of the head and neck is cut away with forceps and scissors, exposing the skull. The skull is grasped in the orbits with mouse-tooth forceps and the calvaria of the skull is cut away with curved scissors, thereby exposing the brain. The brain is removed with curved scissors and is transferred to a sterile Petri dish. A thin section of the brain is cut out, just anterior to the cerebellum, and is transferred to a wooden tongue-depressor or a paper towel. A clean microscope slide is then pressed lightly against the cut surface of the section ; the pressure should be sufficient to create a slight spread of the exposed surface against the glass slide. Negri-body stain and immunofluorescent antibody stain should then be applied to the slide.

Complications of the Mouse Inoculation Test

Bacterial contamination of inoculum

If bacterial contaminants have caused the death of the mice in spite of the addition of antibiotics, and if the original suspension has been preserved, the following methods may be tried in an effort to overcome the interfering action of the bacteria.

(*a*) *Filtration through bacteria-retaining filters :* The supernatant liquid of a suspension centrifuged at 400 *g* for 15 minutes should be used for this purpose. Since rabies virus is a fairly large particle and since, in general, the concentration of virus in specimens submitted from the field is not very great, the virus may be lost in the process of filtration.

(*b*) *Dilution method :* Sometimes the suspension can be diluted beyond the endpoint of bacterial contamination with retention of viral activity, but this happens very rarely.

(*c*) *Prolonged storage :* In some instances, it is easier to combat bacterial contamination after the tissue suspension has been stored for a period of time either at freezing temperatures or in glycerol (see pages 51 and 87).

(*d*) *Parenteral inoculation :* The Syrian hamster, which is the animal most susceptible to parenteral infection, may be chosen for this purpose. Mice are relatively insusceptible to parenteral infection with rabies.

Presence of two viruses

This is particularly confusing if the second virus has pathogenic properties similar to those of rabies. Again, intracerebral or parenteral inoculation of animal species other than the mouse may be attempted, particularly in view of the extremely wide host-range of rabies virus.

Interference phenomena

In certain instances, either because of the properties of a particular strain of rabies virus or because large amounts of inactive virus particles, serum antibody from blood vessels, or other unidentified factors may interfere with the living virus, it may be necessary to dilute the inoculum 10–100 times, or even more. There is no rule for determining when this should be done. However, if failure to isolate rabies virus is consistently encountered in the same species of animal in a particular geographical area, the possibility of an interference phenomenon should be seriously considered, and tissue suspensions should be diluted beyond 10% concentrations.

THE VIRUS NEUTRALIZATION INDEX TEST IN MICE

H. N. JOHNSON [1]

In order to identify an unknown virus by the virus neutralization index test, the virus is titrated in normal serum and in a known virus-immune serum. If the unknown virus is neutralized by rabies-immune serum and not by the normal control serum, it is assumed that the virus under test is a strain of rabies virus. It is important to check all standard stocks of rabies virus by the virus neutralization index test to determine whether the stock virus contains viral contaminants derived from the animals or tissue culture systems used as a source of the virus.

Preparation of Stock Virus Suspension

Prepare a 20% suspension of infected mouse brain using a diluent of phosphate-buffered saline containing 0.75% of bovine albumin fraction 5, or 2% of inactivated guineapig, hamster, or horse serum. The source of the virus is usually the 2nd mouse passage; if this passage is made by intracerebral inoculation of 1–3-day old suckling mice, the virus titre is usually higher than that observed using adult mice. Place aliquots of 0.5 ml or 1.0 ml in 2-ml ampoules, flame seal, and store at −60°C as stock virus. Prepare serial ten-fold dilutions of this stock virus in a diluent as described above, ending with a dilution of 10^{-7}, and titrate each dilution by intracerebral inoculation into mice in order to determine the dilutions of the virus to be tested in the virus neutralization index test.

Serum Specimens

It is essential to use a rabies-immune serum of known potency, previously tested against the virus to show that it possesses specific neutralizing antibody.[2] The standard rabies-immune serum should be stored in the lyophilized state at 4°C or in the frozen state at −15°C or lower in a mechanical freezer. Under such conditions it is not necessary or desirable to use a preservative in the serum. For preparing stock normal and rabies-immune serum, it is best to use hamsters or guineapigs as a source of serum. It is also desirable to use homologous infected brain tissue as the source of antigen in immunizing the animals—for example, infected hamster brain tissue for immunization of hamsters. The virus cultivated in hamster kidney tissue culture may be used for the immunization of hamsters, but if the antiserum is to be tagged with fluorescein it is best to use brain tissue as the source of the antigen. Immunization of the animals for the preparation of rabies-immune serum is usually accomplished by giving 4 weekly intraperitoneal injections of β-propiolactone-inactivated rabies CVS strain brain-tissue virus, mixed with an equal volume of adjuvant. Subsequently, 2 injections of active virus are given at 2-week intervals and the animals are then bled 10 days to 2 weeks after the last dose of virus.

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[2] A known positive serum will be supplied on request by the World Health Organization, 1211 Geneva 27, Switzerland.

Performance of the Test

Serum

Prepare 2 rows of 13- × 100-mm test tubes, to include tenfold dilutions of 10^{-2} through the expected endpoint of the virus. Prepare typed adhesive labels reading N-2, N-3, etc., for the normal serum series, and RI-2, RI-3, etc., for the rabies-immune serum series. In each of the tubes marked N, place 0.2 ml of the normal serum, measured from a 1-ml pipette, graduated in divisions of 0.01 ml. In each of the tubes marked RI, place 0.2 ml of the rabies immune serum. Use a separate pipette for each serum.

Virus

In order to obtain final dilutions of 10^{-2}, 10^{-3} etc. in the serum-virus mixture, prepare tenfold dilutions (2×10^{-2}, 2×10^{-3} etc.) of the stock 20% virus. When mixed with equal quantities of serum, these will give the correct final dilutions. For preparation of the serial dilutions, it is convenient to use 13- × 100-mm test tubes with screw-cap closures. The screw-cap can be handled without contaminating the palm of the hand. Prepare typed adhesive labels reading St-10-2, St-10-3 etc., the prefix St signifying stock virus. Transfer 2.7 ml of the diluent to each of the tubes labelled St-10-2, St-10-3 etc. To prepare the virus dilutions, transfer 0.3 ml of the stock 20% virus suspension to the tube labelled St-10-2. Discard the pipette. Take a clean 1-ml pipette and mix the suspension by filling and emptying the pipette 10 times ; transfer 0.3 ml to the next tube, labelled St-10-3, and discard the pipette. Subsequently, mix and transfer in this manner until the series is complete, using a clean pipette for each dilution. A routine that this mixture is to be pipetted before transfer to the next tube. The pipettes should be filled and the transfer done with a Propipette or a similar pipetting device. Mouth pipetting is dangerous.

Serum-virus mixture

Beginning with the highest tenfold dilution of the stock virus, transfer 0.2 ml to the normal (N) tube and 0.2 ml to the rabies-immune (RI) tube bearing the same dilution number, in that order. Taking a clean pipette, transfer 0.2 ml of the next tenfold dilution to the serum tubes bearing the same dilution number, and so on until the series has been completed. Agitate the serum-virus mixture gently and place it in an incubator at 35–37°C for one hour. Thereafter, transfer the rack to a refrigerator at 4°C to chill the serum-virus mixture quickly and hold it at this temperature until required for inoculation into mice. It is not necessary to keep the serum-virus mixtures in an ice-bath during the inoculation procedure, but it is important to avoid exposing them to sunlight or to intense electric light.

Inoculation of Mice

Inoculate each of 4–6 young adult mice intracerebrally with each serum-virus mixture, using the technique described in chapter 7 (page 89). Complete the inoculation of the rabies-immune serum-virus before testing the normal serum-virus mixtures. A different syringe and needle should be used for each serum-virus mixture.

Interpretation of Results

The usual observation period for the inoculated mice is 21 days but it is recommended that the mice be held for 30 days because of the long incubation periods observed with some natural strains of rabies virus. The titre of the virus in normal serum and rabies-immune serum is calculated as described in Appendix 1 (page 321). The virus-neutralization index is obtained by subtracting the log of the LD_{50} titre of the virus in rabies-immune serum from that in normal serum. If the difference is greater than 2, i.e., if more than 100 times the LD_{50} of the virus is neutralized by the rabies-immune serum, the identity of the unknown virus is established. Mouse-passed strains of brain-fixed rabies virus may sometimes be contaminated with certain natural viruses derived from mice, notably mouse encephalomyelitis virus. For example, the rabies-immune serum will neutralize the rabies virus but not an associated virus derived from the mice. Therefore, if the unknown virus is suspected to be that of rabies and the mice inoculated with the higher dilutions of the virus mixed with rabies-immune serum die, it is necessary to do a specificity test on the brains of the mice, using the fluorescent antibody test (see chapter 6).

Maximum neutralization of most viruses is usually obtained in tests using fresh serum specimens or serum specimens stored in the frozen or lyophilized state. In large-scale testing of serum specimens from dogs vaccinated against rabies, the author has noted a fairly uniform log difference of 1–1.5 in tests of fresh serum and serum inactivated at 56°C for 30 minutes. Inactivation of serum specimens has come into use to avoid differences observed in tests of the same serum when tested fresh and after storage. Rabies hyperimmune serum as used for identification purposes has usually been stored for some time and the blood serum enzymes have been inactivated, so that it is not necessary or desirable to inactivate the serum.

Strains of rabies virus derived from spotted skunks and bats in North America are very pathogenic for infant mice when inoculated intraperitoneally. The pathogenicity is best preserved by isolation and passage in hamster kidney tissue culture, but 1st or 2nd mouse-passage virus is usually pathogenic for infant mice when inoculated intraperitoneally. Virus neutralization index tests using this experimental host system are much more sensitive than the usual mouse inoculation test for detecting virus-neutralizing antibody in blood-serum specimens.

A different neutralization test is used for determining the potency of rabies immune serum. In this test, a constant amount of virus is tested against serial dilutions of serum (see chapter 40, page 314 and Appendix 1, page 321).

PART III

SPECIAL DIAGNOSTIC AND RESEARCH TECHNIQUES

CHAPTER 9

TISSUE CULTURE METHODS

T. J. WIKTOR [1]

The development of improved methods for cultivating animal cells *in vitro* has contributed greatly to the progress of animal virology in general ; it is only during the last few years, however, that these modern techniques have been adapted and used routinely for the study of rabies virus.

The cultivation of rabies virus in tissue culture is extremely important, not only as a means of obtaining knowledge about the virus itself but also as a method for producing large quantities of rabies virus for the preparation of vaccine.

Shortly after tissue culture techniques were first described, they were applied to the study of rabies virus. Levaditi (1913) reported the first successful propagation of rabies virus in spinal ganglia maintained in a medium containing coagulated monkey plasma. Stoel (1930) and Webster & Clow (1937) carried a strain of the virus through several passages in a suspension of chicken or mouse embryo brain without loss of infectivity.

The studies with primary cell explants did not produce much information about the kinetics of virus replication. The development of several cell lines and cell strains susceptible to rabies virus infection has made possible a systematized approach to the study of rabies virus and its interaction with host cells.

New techniques for virus assay have been devised. Immunofluorescent antibody staining for the detection of viral antigen within infected cells (Goldwasser & Kissling, 1958; Kaplan, Forsek & Koprowski, 1960) and the plaque assay technique for testing infectivity (Sedwick & Wiktor, 1967) have led to a better understanding of the mechanism of rabies virus infection.

The adaptation of several virus strains to different tissue culture systems opened the way to a series of investigations on the relationship between cells and rabies virus, revealing a nonlytic, chronic, or endosymbiotic type of infection (Fernandes et al., 1964; Wiktor et al., 1964). Interferon could be detected in rabies-infected tissue culture fluid, and it was found that the production of interferon plays a role in the induction of carrier-type cultures (Wiktor & Koprowski, 1967). The chemical composition of the intracytoplasmic inclusions observed in rabies-infected tissue culture cells tron microscopy techniques have shown that some strains of the virus replicate by budding on the cell plasma membrane, whereas other strains replicate in association with an intracellular cytoplasmic matrix (Hummeler et al., 1967, 1968).

A plaque assay for the quantification and cloning of rabies virus was developed (Sedwick & Wiktor, 1967). Virions propagated in tissue culture have been found to agglutinate goose erythrocytes (Halonen et al., 1968 ; Kuwert et al., 1968). The study of rabies replication in tissue culture systems has permitted the large-scale preparation of cloned populations of the virus that, after concentration and purification, yield higher titres of pure virus for analytical study than can be obtained from the brains of infected animals (Sokol et al., 1968). Disruption of virions propagated in tissue culture has yielded separate components, such as the virus coat proteins and the nucleocapsid, and it has been shown that the virus contains single-stranded RNA (Sokol et al., 1969).

It has been demonstrated that immune antirabies sera lyse infected tissue culture cells in the presence of complement (Wiktor et al., 1968). The kinetics of the formation of this cytolytic antibody differ from those of the neutralizing antibody in immunized animals.

Concentrated, purified, and inactivated virions of tissue culture origin were used experimentally for the immunization of animals (Wiktor et al., 1969). These preparations were several hundred times more antigenic than a standard reference vaccine; they induced extremely high levels of neutralizing antibodies in vaccinated animals and a single dose of vaccine administered after a challenge with a street rabies virus protected the experimental animals (Sikes et al., 1971).

A noninfectious product of virus replication in tissue culture has been separated from the infected fluid by differential ultrafiltration or high-speed centrifugation (Schlumberger et al., 1970; Wiktor et al., 1969). This product has induced antibodies in animals and protected them from challenge, thus opening a new approach to rabies immunization.

It is impossible here to discuss all aspects of the various tissue culture techniques ; only those aspects directly related to the study of rabies virus will be described.

Tissue Culture Systems and Propagation of Virus

Primary cultures

Chicken fibroblasts

Primary embryonic chicken fibroblasts are very susceptible to rabies virus and many strains of fixed and street virus have been propagated in these cells ; however, the system suffers from the disadvantage that most chick embryos are infected with other viruses primarily of the lymphomatosis group, and many also carry mycoplasma. These contaminants can interfere with the growth of rabies virus and produce inconsistent results (Cohen et al., 1963). The possibility that interferon is produced by rabies virus in chick embryo fibroblasts has been suggested by the fact that rabies prevents plaque formation by Western equine encephalitis virus in this system (Kaplan, Wecker et al., 1960).

LEP and HEP Flury virus grown in this system are used for the immunization of animals (Cabasso, Stebbins et al., 1965 ; Cabasso, Sharpless & Douglas, 1965) and the HEP vaccine has also been used experimentally for the immunization of man (Ruegsegger & Sharpless, 1962).

Primary mammalian kidney-cell cultures

The susceptibility of mouse kidney-cell cultures to infection with rabies virus was reported by Vieuchange et al. (1956). Subsequently, Kissling (1958) was able to perform serial passage of both street and fixed virus in hamster kidney cultures. A rabies vaccine for human use propagated in primary hamster kidney-cell cultures has been described (Fenje, 1960).

A strain of virus originally adapted to hamster kidney-cell cultures after a series of passages on embryonated chicken eggs was propagated in pig kidney-cell cultures (Abelseth, 1964a) and is now widely used as ERA vaccine for animal immunization (Abelseth, 1964b).

After adaptation to a human diploid cell strain, the PM virus was adapted to and propagated in primary monkey kidney-cell cultures (Lang et al., 1969). A concentrated and purified vaccine prepared from this virus was used for experimental vaccination of animals (monkeys, rabbits, and mice) and exhibited an extremely high degree of immunogenicity (Sikes et al., 1971).

Continuous cell lines

Several cell lines support the growth of rabies virus. Cell lines possess several advantages over primary cultures ; they can be propagated more easily, characterized to determine that they are free of contaminants, and stored in a frozen state for long-term use. Virus propagated in cell lines, however, is not now considered acceptable for the immunization of man.

Mice ependymoma cell line

This cell line was used by Atanasiu & Laurent (1957) for the cultivation of rabies virus. After passages of street and fixed rabies virus, an inconsistent cytopathic effect was observed and intracytoplasmic inclusions resembling Negri bodies were described for the first time in this cell culture system.

BHK-21 line of hamster kidney fibroblasts

Because of the extreme susceptibility of this cell line to a variety of viruses (MacPherson & Stocker, 1962), it has become the most favoured

(Sokol et al., 1968), for the detection of the haemagglutinating activity of rabies virus (Halonen et al., 1968; Kuwert et al., 1968), and for electron microscopy studies of the mode of intracellular virus replication and of the morphology of the virions (Hummeler et al., 1967, 1968). Basic investigations of the antigenicity of concentrated and purified vaccines have been conducted with BHK-propagated virus (Wiktor et al., 1969). Finally, the first reliable plaquing assay system for rabies virus was developed with a subline of BHK cells (S13) adapted to growth in suspension (Sedwick & Wiktor, 1967).

Rabbit endothelium cell line

Rabies virus can be propagated in this cell line for a prolonged, if not indefinite, period without interfering with the mechanism of cellular replication (Fernandes et al., 1964), even though all cells become infected and those cells undergoing division show the presence of viral antigen throughout the mitotic process. Antirabies serum does not interfere with cell-to-cell transmission of the virus. When exposed to antirabies serum and complement, infected cells undergo complete lysis.

Dog kidney cell line

An epithelial line of dog kidney cells has effectively supported the growth of different strains of rabies virus (Wiktor, unpublished findings). A chronic infection could be established with only some of the cells showing the presence of specific fluorescing inclusions. Flury HEP strains grown in these cells have proved highly antigenic and they are now used for immunization of animals (Brown et al., 1967).

Lower vertebrate cell lines

Rabies virus has been successfully propagated by serial passage in three reptile cell lines: two *Vipera* (snake) cell lines, VSM and 2VH, and one *Gekko* (lizard) cell line, GLI. The viper cell line VSM was particularly susceptible to rabies virus infection, yielding titres of infectious virus (up to 1×10^7 PFU/ml) comparable to those obtained in many mammalian cell lines (Clark, 1972).

Human diploid strains (HDCS)

Various strains of rabies virus have been adapted for growth in this cell system (Hayflick et al., 1961; Wiktor et al., 1964) by means of the cell-mixing technique (see below). Both live and inactivated vaccines prepared in the diploid cell strains have been found highly antigenic for experimental animals (Wiktor & Koprowski, 1965; Sikes et al., 1971). Studies are under way to determine the immunogenicity of this type of vaccine in man. It is expected that a concentrated, purified, and inactivated rabies vaccine propagated in HDCS cells will replace the presently used vaccines for human rabies immunization.

Adaptation of rabies virus to growth in different cell systems

It has been observed with HDCS and several other cell lines (Wiktor et al., 1964) that infection of monolayers or dispersed cell cultures with a virus of brain or salivary gland origin induces fluorescence of a very small number of cells, and that serial transfer of either the supernatant medium or cell extracts in a homologous tissue culture system often results in a gradual decrease of infection. As a rule, after a few passages in HDCS cells, the virus is completely lost.

Infected cultures, maintained in an actively growing state by regular trypsinization and division every 3 to 4 days when the cell sheet becomes confluent, show a gradual increase in the number of fluorescing cells. After a few such divisions (from 4 to 10, depending on the cell system and the virus strain), all cells show fluorescence and the presence of intracytoplasmic inclusions.

After a certain number of subcultures in some tissues, such as HDCS, the cells stop dividing and virus passage can be effected only by mixing a portion of the infected cells with a non-infected homologous cell population at each transfer passage (the cell-mixing technique).

In most cases, after about 40 passages, the virus is sufficiently adapted to growth in a given cell system and can be propagated by using either the supernatant medium or cell extracts for the infection of new cells.

Adsorption and penetration of virus can be enhanced by the presence of polyions, diethylaminoethyl-dextran (DEAE-dextran), and protamine sulfate (Kaplan et al., 1967). The susceptibility of any tissue culture system to infection with rabies virus will improve when the virus is adsorbed in the presence of DEAE-dextran (50 µg/ml) and protamine sulfate (1000 µg/ml). The use of polyions at each transfer facilitates indefinite propagation of the virus in any tissue culture system.

Conditions of cells in culture may alter their susceptibility to rabies. Cells that have been irradiated by ultraviolet rays are much more susceptible to rabies infection than viable cells (Kaplan et al., 1967). From the standpoint of safety, this method of virus growth, if adapted to commercial production, may have certain advantages.

Methods of virus propagation

All conventional methods can be employed for rabies virus propagation in tissue culture. Monolayers in stationary or revolving vessels, as well as in suspension cultures, have been used successfully. The propagation medium is usually Earle's salt solution supplemented with Eagle's amino acids and vitamins or with lactalbumin hydrolysate.

The presence of serum or serum albumin seems to protect against thermal inactivation of the virus, rather than providing the necessary pH (7.4–7.6) by the addition of extra sodium bicarbonate.

Optimum growth is obtained at a low incubation temperature (32°C) (Kissling, 1958 ; Wiktor, unpublished findings) although rabies virus will replicate in the range of temperatures between 20° and 40°C.

Virus Titration and Cloning

Until recently, mouse inoculation was the only method used for the isolation of virus and the quantitative determination of virus infectivity. Several other procedures are now available.

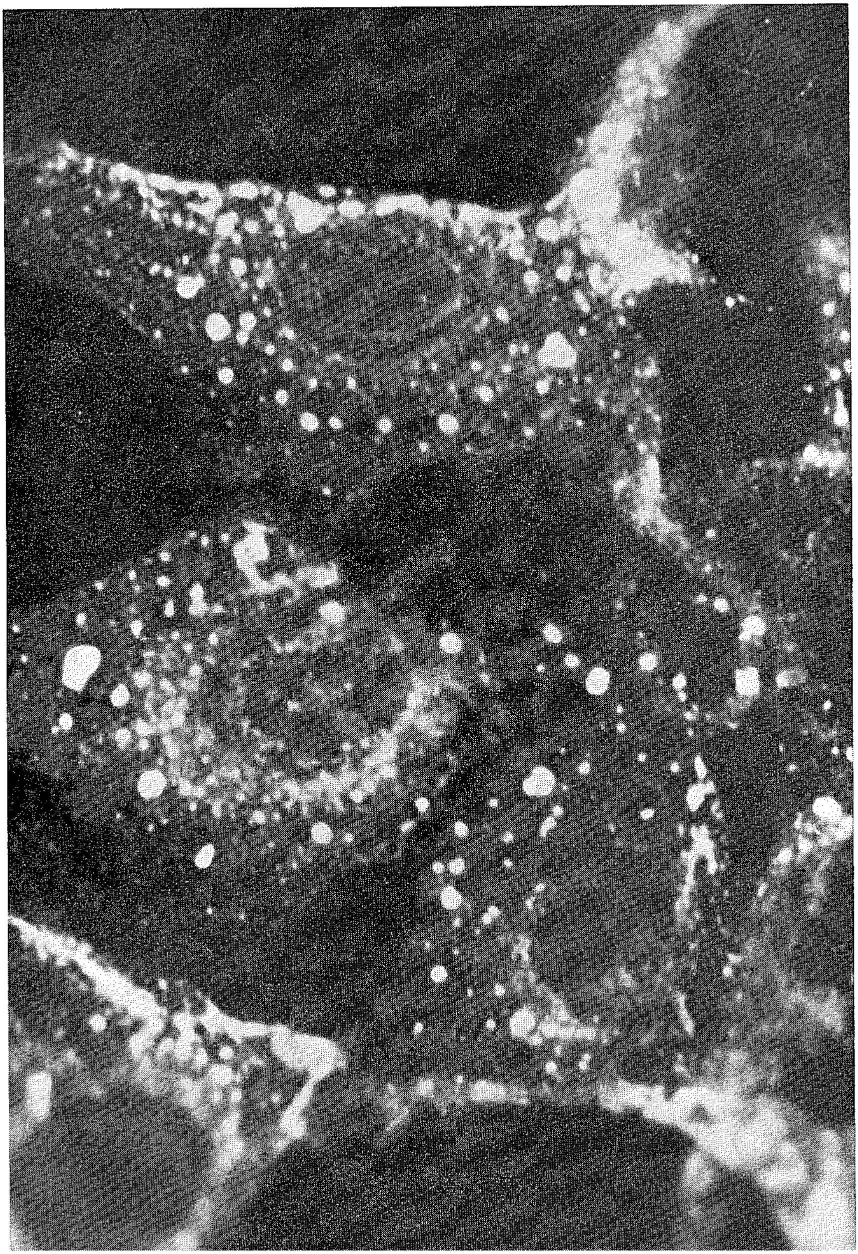
Because a cytopathic effect cannot be produced regularly in rabies-infected tissue culture it is impossible to titrate the virus by direct morphological observation of the infected culture. Staining infected cells by fluorescing antibody for detection of intracytoplasmic inclusions permits a determination of infectivity endpoints.

To detect viral antigen by the presence of intracytoplasmic inclusions, cells should be grown in Petri dishes or tubes containing coverslips. Serial dilutions of virus are prepared in tissue culture medium and an aliquot of each dilution (0.5 ml) is mixed with 5 ml of the cell suspension and poured into Petri dishes containing coverslips. The concentration of cells is adjusted so that a confluent monolayer is obtained after 3–4 days of incubation. At the end of the incubation period (5–6 days after infection), coverslips are removed and stained with fluorescing antibody conjugate or May-Grünwald stain. The highest dilution of virus causing fluorescence of cells or inducing the formation of cytoplasmic inclusions is considered the titration endpoint. However, this method gives only an approximate value of viral infectivity ; for more accurate determination the plaque assay technique is used (see below).

Fluorescent antibody staining

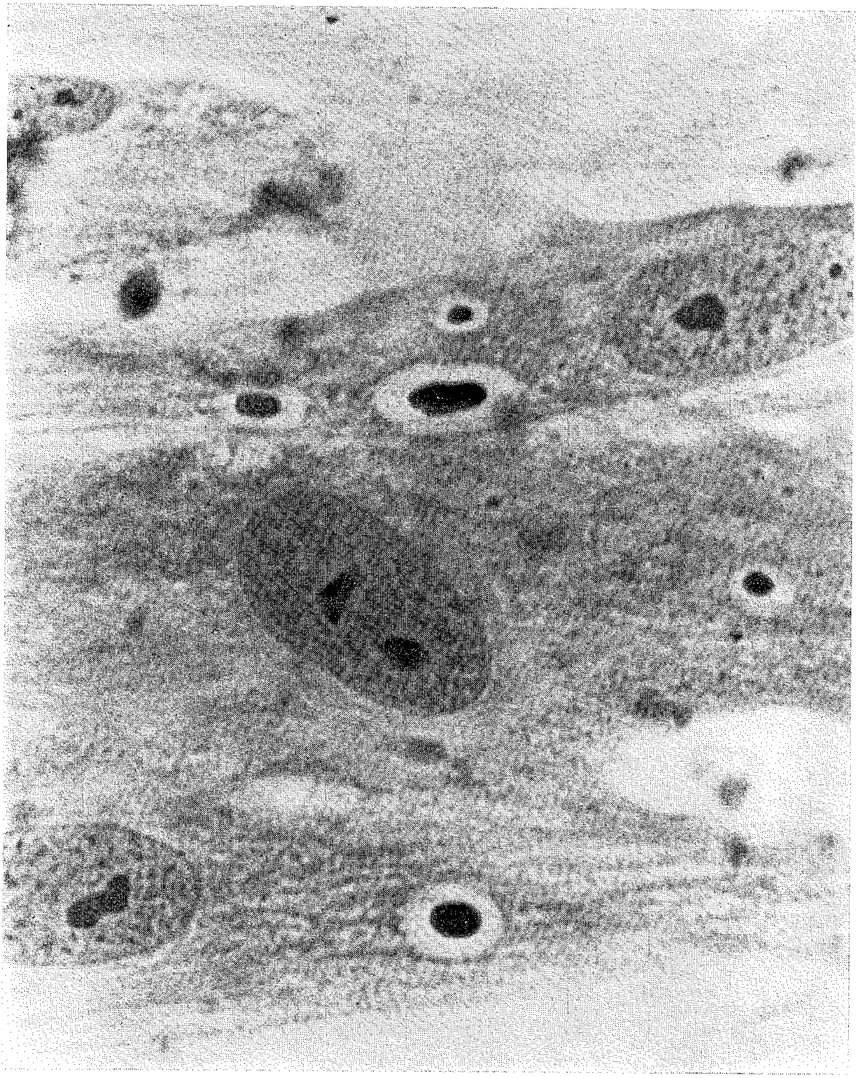
The technique described in chapter 6, page 73, can be used for fluorescent antibody staining of tissue culture preparations. Coverslips with infected cells are removed and placed in the staining racks where they are rapidly washed in phosphate-buffered saline (PBS) and fixed in cold acetone (−20°C for 30 minutes). After fixation they are dried and placed in individual Petri dishes and covered with a few drops of conjugate. The preparations are incubated for 30 minutes at 37°C and then washed for 5 minutes in two changes of PBS and one change of distilled water, dried, and mounted in Elvanol (polyvinyl alcohol) semi-permanent mounting medium (Rodriguez & Deinhardt, 1960) (see Fig. 1). Control preparations, including non-infected cultures and an infected culture stained with the conjugate previously adsorbed on rabies-infected mouse brain in suspension, should be included in the test.

FIG. 1. DEMONSTRATION OF RABIES ANTIGEN BY THE FLUORESCENT ANTIBODY STAINING TECHNIQUE IN HAMSTER CELLS INFECTED WITH THE PM STRAIN OF RABIES VIRUS



Although general techniques used for staining Negri bodies (see chapters 4 & 5, pages 41 & 56) can be used for the detection of intracytoplasmic inclusions in tissue culture preparations, staining with May-Grünwald-Giemsa stain has been found to give the most consistent results (see Fig. 2).

FIG. 2. DEMONSTRATION OF RABIES INCLUSIONS IN WI-38 HUMAN DIPLOID CELLS INFECTED WITH THE FLURY HEP STRAIN OF RABIES VIRUS



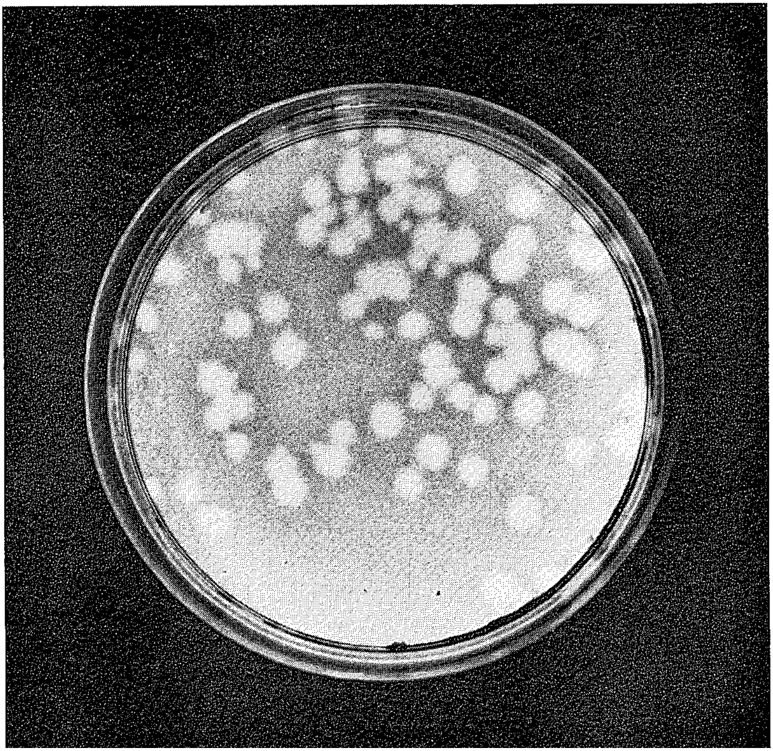
May-Grünwald-Giemsa stain

Preparations are washed in PBS for 2 minutes, then fixed in Bouin fixative for 20 minutes. After being washed in 70% ethanol for 20 minutes, they are stained in May-Grünwald stain for 15 minutes. The May-Grünwald stain is then removed and the preparations are covered with Giemsa stain (1 : 10) for 30 minutes. If necessary, the preparations are differentiated in a 0.5% solution of acetic acid for a few seconds. They are washed with water and treated successively with acetone, a 50% mixture of acetone and xylol, and pure xylol, 2 minutes in each solvent. Finally, they are mounted on slides with Canada balsam.

Plaquing of rabies virus

A technique for the plaquing of rabies virus in agarose-suspended BHK-S13 cell cultures has been described by Sedwick & Wiktor (1967).

FIG. 3. PLAQUES FORMED BY RABIES VIRUS (FLURY HEP STRAIN) IN AGAROSE-SUSPENDED BHK-S13 CELL CULTURES



tests :

Cloning
Virus titration
Evaluation of serum-antibody content by the virus neutralization technique
Evaluation of the antigenicity of inactivated rabies vaccines by the antibody binding test (see chapter 35, page 292).

The classical plaque assay method for animal viruses, as described by Dulbecco (1952), involves adsorption of the virus on a monolayer culture of susceptible cells, followed by the addition of a nutrient agar medium, which becomes solid when cooled. Plaques are visible when the cells are stained with neutral red after an appropriate incubation time (see Fig. 3).

Although plaque formation of rabies virus in chick monolayers has been reported (Yoshino et al., 1966), this technique has not provided consistent results in most laboratories. The ability of BHK-S13 cells to remain viable for a long time when suspended in agarose, and to support the growth of rabies virus, provides a convenient and reliable method for plaquing of rabies virus. The technique follows the method described by Cooper (1961) in which a thin layer of agarose-suspended cells is poured over a layer of nutrient agarose. The virus inoculum is deposited on the top of the cell layer, and after the necessary time of incubation (usually 6 days at 35°C), plaques are counted after staining with neutral red. The cultures are incubated in an atmosphere of 3–5% CO_2 in air.

PLAQUE-FORMING METHOD

The requirements for this method are as follows :

1. *Medium for the propagation of BHK-S13 cells*

Minimum Essential Medium (Eagle) as modified by MacPherson & Stocker (1962) (see formula, Annex 1, page 120) supplemented with :

tryptose phosphate broth	10 %
fetal calf serum (heated for 30 mintues at 56°C)	10 %
penicillin	100 IU/ml
streptomycin	100 µg/ml (78 IU/ml)

2. *Medium for the suspension of the cells*

Minimum Essential Medium, as above, supplemented with :

tryptose phosphate broth	10 %
fetal calf serum (heated for 30 minutes at 56°C)	2 %
penicillin and streptomycin	100 IU/ml

3. *Medium for agarose*

Basal Medium (Eagle, 1955) in a double concentration, without phenol red (see Annex 2, page 121), supplemented with :

fetal calf serum (inactivated)	4 %
penicillin	200 IU/ml
streptomycin	200 µg/ml (156 IU/ml)
mycostatin	100 IU/ml

4. *Agarose stock*

Agarose "Seakem", Bausch & Lomb, 1% in distilled water (sterile). Dissolve by boiling and continue boiling for one hour. (Autoclaving may affect the gelling properties.)

5. *Cells*

A stock of BHK-S13 cells (Vaheri et al., 1965) obtained by serial propagation of BHK-21 cells (MacPherson & Stoker, 1962) in suspension cultures, is maintained frozen in liquid nitrogen for plaque assay ; cells are propagated in monolayer cultures and subcultivated by regular trypsinization. An inoculum of 2×10^7 cells for each 1-litre Roux or Blake bottle will provide a yield of $1.2–1.5 \times 10^8$ cells after 3 days' incubation at 37°C. About 30 subcultivations can be made from the frozen stock ; after this, the ability of cells to produce plaques may be affected.

6. *Petri dishes*

100 glass or plastic Petri dishes, 60 × 15 mm. The bacteriological type of plastic dishes can be used.

7. *Water baths*

Three water baths are required, one with boiling water, one at 42°C, and one at 37°C.

The procedure may be divided into the following steps (the quantities are those required to make 100 plates) :

1. Melt 250 ml of agarose stock in the boiling water bath and when dissolved keep at 42°C.

2. Place 250 ml of medium for agarose in a water bath at 37°C.

3. Trysinize three 1-litre Blake bottle cultures of BHK-S13 and resuspend the cells in medium for suspension of cells, using 10 ml of medium per bottle.

4. Count the cells and adjust the concentration to 8×10^6 cells/ml using the medium for suspension of cells. 50 ml of cell suspension are necessary

250 ml of agarose stock (from the 42°C water bath) and immediately pipette 4-ml aliquots of this mixture into Petri dishes. Replace any unused medium in the 42°C water bath. Allow to harden on a level surface. Final concentration of agarose, 0.5%.

6. Mix 50 ml of cell suspension (see step 4) and 50 ml of agarose medium (see step 5) and pipette 1 ml of this mixture per plate on to a hardened agarose base. Use a level surface and tilt the plates so that the cell mix flows evenly over the base of agarose. Allow to harden. Final concentration of agarose, 0.25%. The plates can be used immediately or after 24 hours' incubation in a carbon dioxide incubator.

7. Infect the plates by depositing 0.1 ml of inoculum on hardened agarose.

8. Incubate at 35°C for a minimum of 5 days or longer depending on the strain of virus tested. The incubator should have a carbon dioxide concentration of 3–5% and 100% humidity.

9. Pour 0.5% agarose containing 1 : 10 000 neutral red over the plates, using 2 ml per plate and read the plaques after 2–4 hours' incubation. Calculate the number of plaque-forming units (PFU/ml) according to the example in Appendix 1, page 333.

Plaque assay technique for rabies virus can also be performed in the absence of a carbon dioxide incubator with a medium in which the buffering system is provided by *N*-tris-(hydroxy-methyl)-methyl-2-aminoethanesulfonic acid (Tes buffer)[1] (Richter, 1967). The formula for the medium for agarose (see Annex 2, page 121) should then be modified as follows:

sterile demineralized water	590 ml
balanced salt solution, 10-fold concentration	200 ml
amino acids, 100-fold concentration	20 ml
vitamins, 100-fold concentration	20 ml
glutamine, 100-fold concentration	20 ml
Tes 1M	55 ml
sodium hydroxide 1M	56 ml
penicillin and streptomycin 100 000 IU/ml	2 ml
mycostatin 50 000 IU/ml	2 ml
fetal bovine serum	40 ml

The medium for the suspension of the cells is prepared by diluting this medium with an equal volume of sterile demineralized water.

Plates are prepared and infected as described above and can be incubated in any bacteriological incubator. Adequate humidity should be provided to avoid excessive desiccation of the gelled medium.

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[1] Obtainable from Calbiochem, Los Angeles, Calif., USA.

A cell monolayer plaque test, using Sephadex as plaquing medium, has recently been described by Schneider, see Appendix 3, p. 339.

Cloning of rabies virus by the plaque-forming method

Serial tenfold dilutions of virus are prepared and 0.1 ml of each dilution is inoculated on to the surface of BHK-S13 cell cultures suspended in agarose. At least 3 cultures are inoculated with each virus dilution; they are incubated for 6 days in a carbon dioxide incubator. Plates are then stained with a nutrient agarose medium containing neutral red. Plates containing a single clone are selected, and the plaque area containing the cells lysed by the virus is collected by suction with a Pasteur pipette and resuspended in 1 ml of nutrient medium. The content of the tube is dispersed by vigorous pipetting or by sonication. Cells and agarose debris are sedimented by centrifugation for 20 minutes at 700 *g* and the supernatant fluid is collected. Three serial tenfold dilutions are prepared from the supernatant and inoculated into a new series of plates. This procedure can be repeated several times; the progeny of the last cloning is used for preparation of a stock of clone-purified seed virus.

Serum Neutralization by the Plaque-Reduction Technique

The method described in chapter 40, page 293 is used with the following modifications:

Preparation and titration of challenge virus

A stock of tissue-culture-adapted, clone-purified CVS strain of virus, is prepared, dispensed into ampoules, and deep frozen. This virus is carefully titrated by the plaque-forming technique and the dilution that will contain 1000 PFU/ml is determined. To correct the effect of thermal inactivation, samples of diluted virus will be maintained for 90 minutes at 37°C before inoculation into the plates. PBS or tissue culture medium containing 2% of fetal calf serum is used as a diluent.

Serum-virus neutralization

The sera to be tested are inactivated for 30 minutes at 56°C; if a bacterial contamination is suspected, it is necessary to filter the serum sample through a Millipore membrane of 0.22 µm or 0.45 µm porosity.

Serial twofold dilutions of the serum are prepared and to each dilution incubation period 0.1 ml of each serum-virus mixture is inoculated on to at least 2 plates.

A reference serum of known activity and a sample of virus diluted in an equal volume of the diluent should be included in the test.

Calculation and interpretation of results

At the end of the 6-day incubation period, plates are stained by overlaying them with a solution of neutral red and the number of plaques in each plate is counted.

The number of plaques in the control preparation containing the mixture of virus and diluent is counted first. This figure is used for determination of the 50 % plaque reduction point.

The following is a typical example of the serum antibody titration :

Serum dilution	*No. of plaques*	*Mean value*
1 : 100	0, 0, 0	0
1 : 200	2, 3, 4	3
1 : 400	13, 16, 11	13
1 : 800	27, 33, 30	30
1 : 1600	60, 54, 54	56
1 : 3200	52, 56, 53	54
1 : 6400	50, 46, 60	52
Control	50, 46, 60	52

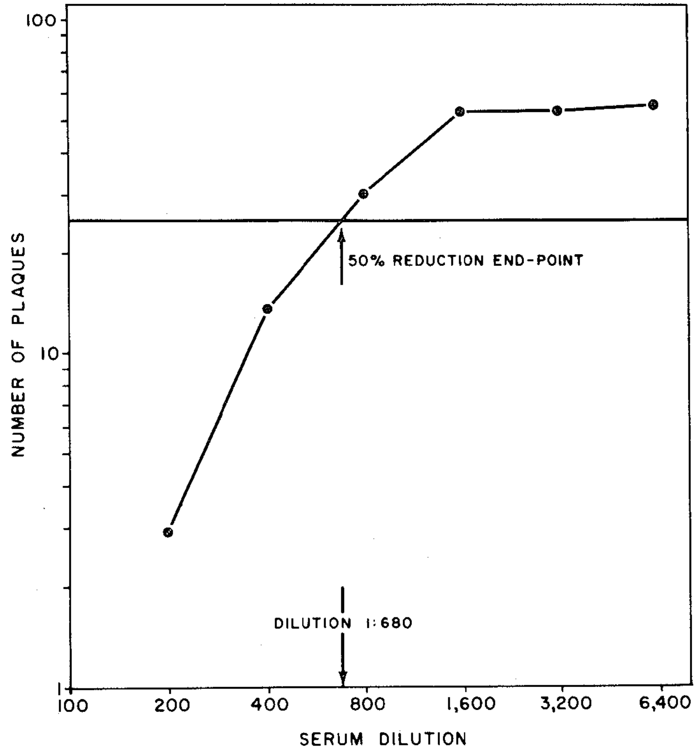
The 50% reduction value in this example is 26 ; thus the titre of this serum will be between the dilutions 1 : 400 and 1 : 800. To determine the exact figure, the mean values for each dilution are determined and the results plotted on semilogarithmic paper (see Fig. 4). The point of intersection of the 26-plaque line with the line obtained by connecting points representing values for each dilution, will indicate the 50% reduction point. In this example, this value is 1 : 680.

Test for Lytic Antibodies

Detection of lytic antibodies by immune cytolysis

Cultures infected with rabies virus are trypsinized, and the cells are then resuspended in the cell suspension medium and reseeded in tissue culture tubes at a concentration of 2×10^5 cells per tube ; alternatively, they may be seeded on coverslips in Petri dishes at a concentration of 1×10^6 cells per 60-mm dish. After incubation for 24–48 hours, the confluent cell monolayers are washed with PBS. When the cells are grown in Petri dishes, individual coverslips are transferred to small (35-mm) plastic dishes prior to the immune cytolysis test.

FIG. 4. DETERMINATION OF THE 50% PLAQUE REDUCTION ENDPOINT IN THE VIRUS NEUTRALIZATION TEST



1 ml of undiluted immune serum, or of the serial twofold or threefold dilution, is added to each tube or dish and incubated for 1 hour at 37°C. The serum is removed and the cells are treated with an appropriate dilution of the complement for an additional hour. The dilution of complement is determined by a preliminary test using a lytic serum with a known titre.

At the end of this incubation period the complement is removed and the cells are stained with trypan blue (0.5% in PBS). The percentage of stained cells is established after counting, under a microscope, a total of at least 1000 cells.

The lytic antibody titre is expressed as the reciprocal of the endpoint dilutions of rabies immune serum causing 10% of the rabies-infected cells

Adsorption and penetration of rabies virus are both extremely rapid. After one second of contact, virus cannot be removed from the cells by washing, and after a minute of contact, some attached virus particles can no longer be neutralized by antirabies serum (Wiktor, 1966).

The dynamics of rabies virus replication and the production of viral antigens can be investigated by immunofluorescence (FA), complement fixation (CF), and haemagglutination (HA) techniques; the production of intracellular and extracellular infectious virus can be evaluated by the plaque assay method.

In a typical experiment, BHK cells were infected with Flury HEP virus at an input multiplicity (IM) of 20, 2, and 0.2 plaque-forming units (PFU) per cell. The virus was adsorbed for 1 hour at 37°C in the presence of 50 μg/ml of DEAE-dextran, and the unadsorbed virus was neutralized with rabies-specific antiserum. The cells were covered with a medium containing 0.3% bovine serum albumin in BME and incubated at 32°C for 8 days. At different intervals after infection, samples were collected for FA, CF, HA, and PFU assay.

The results of these experiments can be summarized, as follows.

Immunofluorescence

At an IM of 20 PFU/cell, immunofluorescence was first observed 8 hours after infection in 24% of the cells. Two hours later, 70 % of the cells showed the presence of FA antigen and this level of infection persisted for the next 6 hours. The second cycle of virus replication apparently started at this time, and 100% of the cells showed FA antigen 24 hours after infection.

At an IM of 2 and 0.2 PFU/cell, FA antigen was also observed 8 hours after infection, but in a smaller number of cells. After several replication cycles, all cells became FA positive at 36 and 48 hours after infection, respectively (see Fig. 5).

Infectious virus

Even though the cells were treated with antirabies antibodies after an adsorption time of 1 hour, a small amount of *residual virus* persisted in the cultures during the first 7 hours after infection at an IM of 20 PFU/cell. No infectivity was detected at the lower IM.

The eclipse phase apparently lasted for 9–12 hours after infection, as determined by an almost 100-fold drop in the infectivity titres of cell-associated virus as compared with the reading at the beginning of

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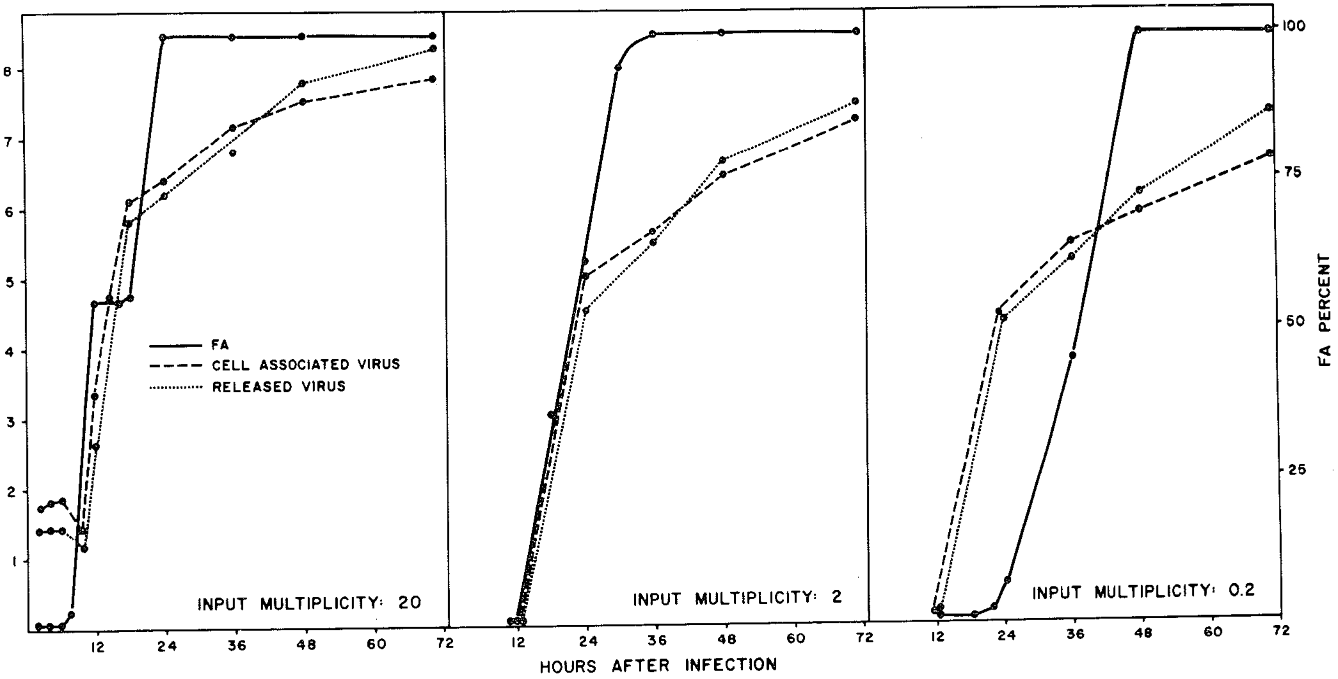
* Based on unpublished experiments performed in collaboration with E. Kuwert.

FIG. 5. KINETICS OF RABIES VIRUS REPLICATION: FLURY HEP STRAIN OF RABIES VIRUS IN BHK-21 CELL CULTURES.



Formation of antigen, demonstrable by the fluorescent antibody staining technique, and production of infectious virus cellular virus was insignificant.

Newly produced virus was detected in the cells as well as in the tissue culture fluid at 12 hours after infection (no samples were taken between 9 and 12 hours). The level of infectious virus increased rapidly during the next 6 hours and continued to rise with prolonged incubation. Initially, more virus was present in the cells than in the medium, but after the 30th hour after infection the medium contained a higher level of infectivity, indicating a continuous production of infectious virions in the cells with subsequent accumulation in the medium.

The IM of infection had no effect on the maximum yield of virus. The peak of infectivity was obtained in 4 days after infection at an IM of 20 PFU/cell while 6 days were required to achieve the same level with an IM of 2 or 0.2 PFU/cell (see Fig. 5).

No specific cytopathic effect could be observed in rabies-infected BHK cultures. By the 8th day of incubation, the infected cells, as well as the control cells, showed a pronounced disintegration as a result of aging.

Haemagglutinin

The dynamics of haemagglutinin (HA) production by rabies-infected cultures parallels the production of infectivity. Practically all HA activity is found in the tissue culture fluid. Cell-associated virus contains very little of this activity despite the fact that, in the early stage of infection, the concentration of infectious virus is higher in the cells than in the tissue culture fluid.

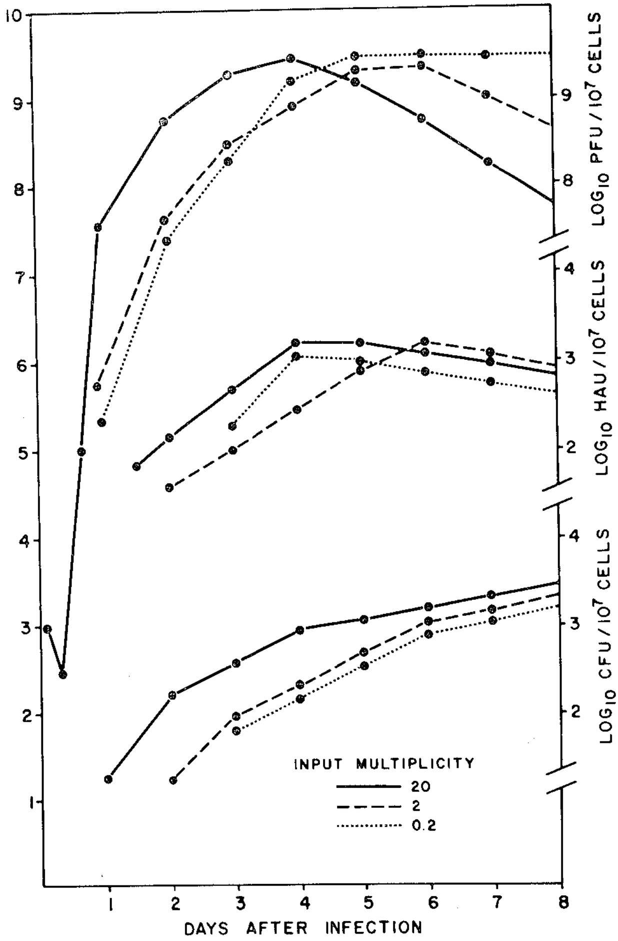
The infectivity of the virus is proportional to the HA activity, provided that the infectivity of the virus sample is higher than or equal to 10^{-6} PFU/ml.

Haemagglutination was first detected 30 hours after infection at an IM of 20 PFU/cell, and on the second and third day after infection at an IM of 2 and 0.2 PFU/cell, respectively. The level of HA increased in parallel with the infectivity; both reached a peak at approximately the same time and had decreased slightly by the end of the 8-day incubation period (see Fig. 6).

Complement-fixing activity

Complement-fixing (CF) antigens in rabies-virus-infected cultures can be detected 24 hours before the appearance of HA. The dynamics of their formation follows the production of infectious virus. Early in the infection, higher activity is observed in the cells; later on, higher activity is detected in the culture fluid. CF activity appears to be more resistant to heat inactivation than HA and infectivity (see Fig. 6).

FIG. 6. KINETICS OF RABIES VIRUS REPLICATION: FLURY HEP STRAIN OF VIRUS IN BHK-21 CELL CULTURES



Production of infectious virus, haemagglutinins and complement-fixing antigen

Byproducts of Viral Infection

Production of virion-free rabies antigen

In addition to infectious virus, cells infected with rabies virus produce noninfectious material, which can be separated from rabies virions (Wiktor et al., 1969).

Virion-free rabies antigen reacts immunologically (in CF and gel-virions. The specificity of the immunological reaction with virion-free rabies antigen is further proved by its ability to protect animals against challenge with infectious virus. This protective ability suggests the possibility of using virion-free rabies antigen in the preparation of a new type of rabies vaccine (Schlumberger et al., 1970).

Production of interferon and induction of chronic cell infection

Rabies-infected cells can be propagated through numerous transfers without showing any cytopathic effect. Production of infectious virus reaches a peak 2–3 days after inoculation and falls sharply in the course of subsequent cell transfers. At the peak of infection, all cells in the culture show the presence of rabies-specific inclusions demonstrable by immunofluorescence. The culture also becomes resistant to challenge with vesicular stomatitis virus, Mengo virus, and poliovirus, as well as to challenge with the homologous or heterologous strains of rabies virus. At this stage of infection, interferon is present in the tissue culture medium. Subsequently, a marked decrease in the number of antigen-containing cells can be observed, followed by a gradual rise in the proportion of infected cells and a new cycle of infectious virus production (Wiktor & Koprowski, 1967).

The cycle of high and low levels of virus infection and corresponding resistance to challenge and interferon production recurs periodically.

When cells from a culture with a low rate of infection are cloned, it is possible to obtain cultures completely free from virus infection, which are as susceptible to reinfection with rabies virus as are control cultures. On the other hand, it is also possible to obtain positive clones undergoing cycles of high and low levels of infection.

Annex 1

MEDIUM FOR PROPAGATION OF CELLS: BHK-21 MEDIUM *

Component	*mg/litre*
sodium chloride	6400.0
potassium chloride	400.0
calcium chloride (anhydrous)	200.0
magnesium sulfate, heptahydrate	200.0
sodium dihydrogen phosphate, monohydrate	124.0
glucose	4500.0

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* MacPherson & Stoker, 1962.

ferric nitrate, nonahydrate	0.10
L-arginine hydrochloride	42.0
L-cystine	24.0
L-histidine	16.0
L-isoleucine	52.0
L-leucine	52.0
L-lysine hydrochloride	74.0
L-phenylalanine	33.0
L-threonine	48.0
L-tryptophan	8.0
L-tyrosine	36.0
L-valine	47.0
L-methionine	15.0
L-glutamine	292.0
i-inositol	3.60
choline chloride	2.0
folic acid	2.0
niacinamide	2.0
calcium D-pantothenate	2.0
pyridoxal hydrochloride	2.0
thiamine hydrochloride	2.0
riboflavin	0.2
phenol red	15.0
sodium bicarbonate	2750.0

Annex 2

MEDIUM FOR AGAROSE: BASAL MEDIUM (EAGLE) *

Final composition of medium

Component	*mg/litre*
sodium chloride	6800.0
potassium chloride	400.0
sodium dihydrogen phosphate, monohydrate	140.0
magnesium sulfate, heptahydrate	200.0
calcium chloride, anhydrous	200.0
glucose	1000.0

Stock solutions[a]

Solution A: Balanced salt solution (Earle's)

10-fold concentrate (without sodium bicarbonate and phenol red)

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* Eagle, 1955.

[a] The stock solutions listed here are available in the form of concentrates: the strengths

L-cystine	12.0	100-fold concentrate
L-tyrosine	18.0	
L-histidine	8.0	
L-isoleucine	26.0	
L-lysine	26.0	
L-methionine	7.50	
L-phenylalanine	16.50	
L-threonine	24.0	
L-tryptophan	4.0	
L-valine	23.50	
biotin	1.0	*Solution C :* Vitamins 100-fold concentrate
folic acid	1.0	
choline chloride	1.0	
nicotinamide	1.0	
calcium D-pantothenate	1.0	
pyridoxal hydrochloride	1.0	
thiamine hydrochloride	1.0	
riboflavin	0.10	
i-inositol	1.80	
L-glutamine	292.0	*Solution D :* Glutamine 100-fold concentrate
sodium bicarbonate	2200.0	*Solution E :* Sodium bicarbonate 5.6 g/100 ml

Quantities required for preparation of a 2-fold concentrate [a] of the medium

sterile demineralized water	616 ml
stock solution A	200 ml
stock solution B	20 ml
stock solution C	20 ml
stock solution D	20 ml
stock solution E	80 ml
penicillin and streptomycin, 100 000 IU/ml	2 ml
mycostatin, 50 000 IU/ml	2 ml
fetal bovine serum	40 ml

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[a] When diluted with an equal volume of 1% agarose in sterile demineralized water, this concentrate gives a solution having the final composition shown above.

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THE COMPLEMENT FIXATION TEST

E. KUWERT[1]

The complement fixation test (CFT), an important and widely accepted technique for the diagnosis of many virus diseases (Fulton, 1958), has not so far proved very useful in rabies research and diagnosis. For the test to be reliable, it is necessary to use pure, concentrated antigen for the demonstration of CF antibody, or monospecific antisera with high antibody titre for the demonstration of CF antigen. Since rabies virus (RV) does not yield reproducible high titres in non-nervous tissue, CF antisera have in the past been produced mainly by immunization of animals with suspensions of CNS tissue containing — on the basis of weight or nitrogen content — very little RV (Kuwert, 1967). The resulting antisera, therefore, exhibited more CF activity against CNS tissue than against rabies virus. Antibodies against brain lipids — for example, cerebrosides (Niedieck et al., 1965) or gangliosides—or against water-soluble brain material interfered with the specificity of the rabies CFT. Treatment of these sera with brain tissue of non-infected animals resulted not only in the adsorption of the organ-specific antibodies but also in a considerable decrease in the CF antibodies against RV.

One successful method of producing RV-specific CF antisera has been found to be the treatment of a CNS suspension from infected animals with chloroform (Kuwert, 1960, 1961a, 1961b, 1967; Kuwert & Bindrich, 1958). Antigens obtained in this way no longer contain infectious virus and can be used to prepare highly RV-specific CF antisera. A considerable advance was made, however, when a highly purified and concentrated RV grown in tissue culture was developed (Sokol et al., 1968; Hummeler et al., 1968) for serum production. After removal of non-specific antibodies by absorption on non-infected tissue cells, sera obtained in this was proved to be exclusively RV-specific and had high CF titres. The CFT, unlike the haemagglutination reaction, measures all virus-specific antigens and not only virions. It can be employed, therefore, for the study of the so-called S-(soluble-)antigens, which have RV antigenicity but are not virion-associated. The sensitivity threshold of the CFT is about 10–20 times that of immunodiffusion.

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There are three main methods of performing the CFT—the macro method, the small volume method, and the micro method. The relative proportions of the reagents used, the reaction temperature, and the reaction time are the same for all three methods. The actual volumes of reagents used are shown in the accompanying table. For measuring the volumes in the micro method, microdiluters (see Chapter 11, Fig. 2), or plastic microdroppers may be used in place of the usual 1.0-ml serological pipette. Plastic plates with 96 indentations or cups each are available for carrying out the micro test; for the macro method and the small volume method, glass tubes are used. All three methods give similar endpoints on visual reading. Only the macro method is suitable for determination of the 50% haemolytic endpoint, if a high degree of accuracy and reproducibility is desired, but the micro method is quite satisfactory for routine use. Both methods are described below. The small volume method has also been found satisfactory and economical and can be recommended, but it will not be described in detail.

VOLUMES OF REAGENTS REQUIRED FOR DIFFERENT METHODS OF PERFORMING THE COMPLEMENT FIXATION TEST

Reagent	Macro method (ml)	Small volume method (ml)	Micro method[1] (ml)	
			(*a*)	(*b*)
Antigen (antibody)	0.2	0.1	0.05	0.025
Complement	0.2	0.1	0.05	0.025
Serum dilution	0.2	0.1	0.05	0.025
Sensitized cells	0.4	0.2	0.1	0.05
Total volume	1.0	0.5	0.25	0.125

[1] For the micro method, the volumes shown in column (*a*) may be used to give increased accuracy if sufficient quantities of antigen or antibody are available. The volumes shown in column (*b*) give satisfactory results for most purposes.

General Considerations

As already explained, the complement fixation test may be used for the quantitative determination of either rabies CF antigen or rabies CF antibody. Before carrying out the test proper, however, it is first necessary to standardize the sheep erythrocytes, haemolysin (amboceptor), and complement used. The sensitivity and specificity of the reaction depend upon the accuracy with which these reagents are standardized with respect to one another (Lennette, 1964). Precision of measurement is therefore of the utmost importance, especially when small volumes are used. Careful standardization of the sheep erythrocyte suspension by determination of the different days will yield comparable values only if the same sheep cell concentration (as indicated by the same optical density at 546 nm) and the same amount of C′ are used on each occasion. In other words, the shape of the response curve of the haemolytic reaction must remain unchanged throughout the period of testing. A detailed discussion of the mathematical basis for the haemolytic assay of complement has been given by Mayer (1961).

Standardization of Haemolysin and Complement

Titration of haemolysin (amboceptor)

The 50% unit of haemolytic antibody (AbH_{50}) is defined as the number of millilitres of sheep erythrocyte antiserum that, in the presence of excess C′ (ten 50% units of $C' = 10\ C'H_{50}$) and optimal Ca^{++} and Mg^{++} concentrations and at an ionic strength of 0.147, will lyse 0.35×10^9 sensitized sheep erythrocytes within 45 minutes in a total reaction volume of 5.0 ml at 37°C. The test has to be performed in glass tubes (macro method).

1. Prepare and standardize sheep erythrocytes as indicated in the annex (p. 131), adjusting the optical density to 0.400.

2. Prepare accurately a series of haemolysin dilutions from 1 : 251 to 1 : 39 800 (for the preparation of haemolysin, see Annex). Starting with a dilution of 1 : 251 (1 ml of the 1 : 100 dilution of haemolysin + 1.51 ml of barbital-buffered saline (BBS, see Annex) and using the dilution factor of 1.59 (log 1.59 = 0.2), 2.0 ml of each dilution are transferred to 1.18 ml of BBS to obtain the next dilution. When the serial dilution process has been completed, discard 0.18 ml from every tube, leaving 1.0 ml in each.

3. Add 2.0 ml of BBS to each haemolysin dilution tube.

4. Add to each tube 1.0 ml of sheep erythrocyte suspension with an O.D. of 0.400 at 546 nm.

5. The haemolysin/erythrocyte mixture is incubated for 30 minutes at 37°C to sensitize the cells.

6. 1.0 ml of C′ is added to each tube. Usually a 1 : 20 dilution of guineapig serum corresponds to 10 $C'H_{50}$.

7. Incubate at 37°C for 45 minutes.

8. Estimate the degree of lysis by visual observation and record the results. Select the tubes with partial lysis for determination of the 50% haemolytic endpoint. Centrifuge these tubes at approx. 500 *g* for 10 minutes, mix the supernatant with five-eighths of the transformation solution (see Annex), allow to stand for 20 minutes and determine the O.D. at 546 nm, using the transformation solution as a blank.

9. Plot the O.D. against the haemolysin dilution on semilogarithmic paper and determine the AbH_{50}. The principle of this graphic determination is illustrated in Fig. 1. The actual value for the AbH_{50} in this example is 1 : 13 000 (= 7.7×10^5 ml of haemolysin). For sensitization of the sheep cells 10 AbH_{50} are used.

Titration of complement (C′)

In order to remove natural haemolytic antibody, pooled guineapig serum is mixed with one-thirtieth of its volume of packed sheep erythrocytes and incubated for 15 minutes in the cold (0–4°C). This treatment is repeated once or twice.

The 50% haemolytic unit of complement ($C'H_{50}$) is defined as the number of millilitres of normal guineapig serum that, in the presence of optimal Ca^{++} and Mg^{++} concentrations and at an ionic strength of 0.147, will lyse 0.035×10^9 optimally sensitized sheep erythrocytes within 45 minutes in a total reaction volume of 1.0 ml at 37°C. The C′ titration must be performed in glass tubes (macro method).

1. Prepare a series of glass tubes. In the first tube place 2.41 ml of BBS and in all following tubes 1.18 ml of BBS. Add 0.1 ml of C′ (see Annex) to the first tube and after thorough mixing transfer 2.0 ml to the next tube. Repeat the transfer serially. Discard 2.98 ml from tube 9, 0.98 ml from tubes 8 to 2, and 0.31 ml from tube 1, so that 0.2 ml of C′ dilution remains in each tube.
2. Add 0.4 ml of BBS to each tube.
3. Add 0.4 ml of haemolytic system (O.D. 0.200) to each tube.
4. Incubate for 45 minutes at 37°C.
5. Centrifuge the tubes exhibiting partial haemolysis.
6. Mix the supernatant (0.75 ml) with 1.25 ml of transformation solution (see Annex).
7. Allow the mixture to stand for 20 minutes and then read the O.D. at 546 nm against the blank transformation solution.
8. Plot the O.D.s against the C′ dilutions on semilogarithmic paper and determine the $C'H_{50}$ graphically.
9. Calculate the C′ dilution (2.0 or 2.5 $C'H_{50}$) for the final CFT (1 $C'H_{50}$ = 1 : 208 ; 2 $C'H_{50}$ = 1 : 104 ; 2.5 $C'H_{50}$ = 1 : 83.2).

CFT for the Titration of RV Antigen or RV Antibody

The CFT depends on competition for C′ between two antigen-antibody systems : (1) the test system, or virological system, consisting of RV antigen and RV antibody ; and (2) the haemolytic or indicator system, consisting of sheep erythrocytes and haemolysin. The reaction is performed in two stages. The first stage consists in incubation of the C′ with the test system only for a period of 45 minutes to 3 hours at 37°C (Wassermann technique) or for 18 hours at 4°C (Kolmer technique). At the end of this first incubation period, the haemolytic system is added and the mixture is then incubated at 37°C for a further 30 minutes. The haemolytic system "indicates" whether RV antigen and RV antibody are present or not. If one of these two components of the test system is absent, no reaction occurs—the C′ will not be "fixed" but will be free to react with the indicator system and haemolysis will occur. Absence or reduction of such haemolysis indicates that the C′ was "fixed" by the RV antigen-antibody combination, provided that the controls show the results specified below in step 10.

Materials

Rabies virus working CF antigen and antiserum

Working CF antigen and antiserum are distributed by the WHO Intercan be stored in this condition for an unlimited time at —20°C or below. Rehydrated antigens should not be kept for more than a week at +4°C. If not needed within 8 days after rehydration they should be stored at —80°C. Repeated freezing and thawing of RV up to 9 times does not impair its infectious and antigenic properties provided the buffer diluent contains 0.4% of bovine serum albumin.

Candidate antigens

These are prepared as 10% tissue suspensions in phosphate-buffered saline, pH 7.0, the tissue being ground in a mortar but without quartz powder. The suspension is extracted at +4°C for 60 minutes and then centrifuged at approx. 3000 *g* for 15 minutes. The supernatant represents the antigen ready for titration without further treatment. RV-containing tissue culture medium can be used as antigen after clarification by centrifugation as indicated above. RV-containing tissue culture cells have to be lysed by ultrasonication and/or repeated freezing and thawing. The cell detritus suspension must also be clarified by centrifugation before titration.

Candidate sera

Sera for evaluation of CF antibody should not contain any anticoagulants, such as EDTA or sodium citrate, which exert a strong anticomplementary effect. They should also be free from haemolytic activity, since haemoglobin may also exert an anticomplementary effect. The sera have to be inactivated for 30 minutes at 56°C before titration.

Micro method

For diagnostic purposes, or if very little antigen or antiserum is available (e.g., in gradient centrifugation experiments), the micro method using as little as 0.025 ml of each component yields satisfactory results.[2] The reliability can be increased by duplicate titrations.

1. Prepare the micro plates by marking the cups to be used for the antigen and antiserum dilutions and for the antigen, antibody, and C′ controls (for preparation of C′, see Annex). The following controls are needed :

(*a*) *Antigen controls with C′* : RV or candidate antigen dilutions tested for anticomplementary activity in the absence of RV antiserum and without addition of the haemolytic system.

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[1] Laboratories wishing to prepare their own CF antiserum should note that the use of rabbits or dogs is mandatory for this purpose as many other animal species have been found to give inconsistent results.

[2] For greater accuracy, double the quantities of reagents may be used, if sufficient antigen or antiserum is available, as indicated in the table on page 125.

(*b*) *Antigen controls without C′* : RV antigen dilutions tested for procomplementary activity in the absence of RV antiserum and without addition of the haemolytic system.

(*c*) *Antibody controls with C′* : RV or candidate serum dilutions tested for anticomplementary activity in the absence of RV antigen and without addition of the haemolytic system.

(*d*) *Antibody controls without C′* : Serum dilutions tested for procomplementary activity in the absence of specific antigen and without addition of the haemolytic system.

(*e*) *C′ controls* : back titration of C′ in the absence of RV antigen and RV antiserum but with the addition of sensitized erythrocytes after the first incubation period.

2. Add 0.025 ml of BBS to each of the cups to be used for antigen (antiserum) dilutions and to each of the cups to be used for antigen, antibody, and C′ (back titration) controls.

3. Add 0.05 ml of BBS to each of the control cups to be used for antigen and antibody without C′.

4. To the first cup of the antigen titration row, add 0.025 ml of candidate antigen (this gives a dilution of 1 : 2). For the titration of antibody, use 0.025 ml of candidate serum instead of antigen. Add the same quantities of antigen or antiserum to the control cups.

5. Prepare serial dilutions by means of 0.025-ml microdiluters or a multimicrodiluter. Dip the diluters in BBS before use and make sure that no air bubbles are present as these might falsify the results.

6. Drop 0.025 ml of CF-RV antiserum (10 AbH_{50}) into each of the cups containing antigen dilution and into the antibody control cups. For the titration of antibody use CF-RV antigen (4–8 units) in place of CF-RV antiserum.

7. Drop 0.025 ml of C′ (2.5 $C'H_{50}$) into all the cups, with the exception of the antigen controls without C′ (for antigen titration) and the serum controls without C′ (for antibody titration).

8. Seal the plate with adhesive plastic tape and incubate for 18 hours at +4°C (Kolmer technique) or for 90 minutes at 37°C (Wassermann technique).

9. Add to each cup 0.05 ml of the suspension of sensitized sheep erythrocytes, seal the plate once more, and incubate for 30 minutes at 37°C.

10. Read the results by means of the Microtiter magnification mirror. The test is valid if the antigen and antibody controls with C′ show complete haemolysis and if there is complete absence of haemolysis in those without

10% haemolysis in the third cup (0.63 $C'H_{50}$). Parallel titration of CF-RV standard antigen or of CF-RV standard antiserum should give the expected values. The last antigen (or serum) dilution exhibiting partial haemolysis is taken as one complement-fixing antigen unit (CF-AGU) or as one complement-fixing antiserum unit (CF-ASU).

Macro method

The use of more than 0.05 ml of each reagent in the CFT may be necessary if precise endpoints are required. The procedure is similar to that described above for the micro method, but the plastic plates are replaced by glass tubes and the microdiluters by conventional pipettes. The quantity of each reagent is increased to 0.2 ml and the total volume of 1.0 ml per tube permits the determination of the 50% haemolysis antigen ($AgC'H_{50}$) or antibody (AbH_{50}) values.

Determination of Optimal RV Antiserum and RV Antigen Concentrations for Checkerboard Titrations of CF Activity

Whereas routine titrations of either CF-RV antigen or CF-RV antibody are performed by the so-called "straight-line" method, for the titration of the CF activity of RV standard antigen and standard antiserum preparations it is mandatory to use the "box" or "checkerboard" system. This is the only method that permits the appropriate concentrations of the 2 standard preparations to be determined precisely, thus making it possible to carry out routine titrations without the risk of errors due to antibody excess or antigen excess (pre-zone phenomenon). Box titration can also be performed in micro plates. The procedure is the same as that described above except that instead of different antigens different dilutions of one antigen and one antiserum and hence different antigen-antibody ratios are tested for their C′-fixing capacity.

If, however, exact antigen or antiserum endpoint values based on the 50% haemolysis are desired, the checkerboard titration has to be performed in glass tubes (macro method) using 0.2-ml volumes of each of the 5 reagents. For each antigen concentration the corresponding 50% haemolytic antiserum endpoint ($ASC'H_{50}$) is then determined and for each antiserum dilution the corresponding antigen value ($AgC'H_{50}$). The results can be integrated into the so-called isofixation curve (Rapport & Graf, 1957), which is the basis for exact comparison of different virus strains by complement-fixation. More detailed information will be found in the papers of Rapport & Graf (1957) and Kuwert (1968).

Annex

PREPARATION, SOURCES, AND STORAGE OF REAGENTS

Alsever's solution

The formula is given in chapter 11, page 143.

Sheep blood

Blood is withdrawn from the jugular vein of an adult sheep. It can be stored for about 4–6 weeks at 4°C in Alsever's solution (2 parts of blood to 1 of Alsever's solution).

Anti-sheep-erythrocyte antibody (haemolysin)

Haemolysin (or amboceptor) is obtained from rabbits immunized with a suspension of sheep erythrocytes or stromata prepared from them as described by Mayer (1961). The rabbit serum is inactivated at 56°C for 30 minutes. A 1 : 100 dilution (1 ml of haemolysin + 1 ml of glycerol in 98 ml of barbital buffer) can be stored indefinitely at −20° to −80°C if divided into small aliquots.

Barbital-buffered saline (BBS)[1]

This is a satisfactory diluent for all the reagents used in the CFT. It has a pH of 7.2.

Solution A

sodium chloride	83.80 g
sodium bicarbonate	2.52 g
barbital sodium (sodium salt of 5,5-diethylbarbituric acid)	3.00 g
distilled water . . . to make	1000.00 ml

Solution B

barbital (5,5-diethylbarbituric acid)	4.60 g
magnesium chloride hexahydrate	1.00 g
calcium chloride dihydrate	0.20 g
hot distilled water . . . to make	500.00 ml

Cool solution B, add solution A and dilute to 2000 ml with distilled water. This gives a 5-fold concentrate. Sterilize by Seitz or Millipore filtration.

Working solution

Dilute 200 ml of the concentrate to 1000 ml with distilled water (1 : 5 dilution).

When the test is performed in glass tubes, there may be some adsorption of C′ on the surface of the glass. To reduce this adsorption, 0.1% of inactivated normal rabbit serum, 0.1% of gelatine, or 0.1% of bovine serum albumin may be added to the barbital working solution. With plastic plates this precaution does not appear to be necessary (Fulton, 1958).

Complement (C′)

Normal serum obtained from 5–10 guineapigs is mixed and stored in 0.5 ml aliquots at −70°C or below. At this temperature the C′ titre does not change over a period of about 12 months. At −20°C, the C′ activity decreases rapidly. Guineapig sera do not contain natural antibody to RV antigen but occasionally contain antibody to sheep erythrocytes. Before the sera can be used in the CFT this antibody must be removed by adsorption on normal sheep erythrocytes (see below).

Transformation solution for determination of the 50% haemolytic endpoint

The 50% haemolytic endpoint in C′, antigen, or antibody titrations is determined by measuring the amount of haemoglobin released from the immunolysed sheep erythrocytes by C′ not fixed in the primary reaction between RV and its antibody. Since haemoglobin is very easily converted into methaemoglobin or sulfhaemoglobin, undergoing pronounced changes in colour and in light absorption properties, it is advisable to form a stable haemoglobin complex that does not change colour, even on prolonged storage. On treatment with cyano compounds, the divalent haemoglobin is converted into the trivalent haemiglobincyanide, which shows a light absorption maximum at 546 nm and is very stable (Mester & Kuwert, 1963). It is prepared by mixing the following reagents :

sodium bicarbonate	1.00 g
potassium ferricyanide ($K_3Fe(CN)_6$)	0.2 g
potassium cyanide	0.5 g
distilled water . . . . to make	1000.00 ml

For determination of the optical density 1.25 ml of this transformation solution is added to 0.75 ml of haemoglobin solution. Erythrocytes that are not lysed have to be removed by centrifugation or simply by sedimentation of the CFT at +4°C overnight.

Sensitized sheep erythrocytes

Sheep erythrocytes, stored in Alsever's solution, are washed 3–4 times with BBS before sensitization by haemolysin. After the last washing the cells are packed in a conical graduated centrifuge tube. To increase the sensitivity of the CFT, instead of the usual 2.0% suspension of packed sheep cells only a 1% suspension in BBS is prepared. To 50 ml of this suspension is added an equal volume of haemolysin, equivalent to 8 amboceptor units. (One amboceptor unit is the amount of anti-sheep-erythrocyte antibody that in the presence of excess C′, will still cause 50% haemolysis of a 0.5% sheep cell suspension). The haemolytic system must have an extinction value of 0.200 at 546 nm as measured against the blank transformation solution. The optical standardization of the erythrocyte suspension is necessary for the reproducibility and accuracy of the CFT. It is performed as follows: 0.75 ml of a 1% sheep cell suspension is lysed by freezing and thawing in a centrifuge tube. After addition of 1.25 ml of the transformation solution the mixture is kept at room temperature for 20 minutes. The resulting haemiglobincyanide solution should have an extinction value of 0.4. If not, this value can be obtained by adjusting the volume according to the formula

$$V_f = V_i \frac{O.D.}{0.4}$$

where O.D. is the optical density of the lysate of the 1% sheep cell suspension, V_i is an accurately measured volume of the approximately 1% suspension, and V_f is the final volume to which it should be diluted by addition of BBS.

The haemolytic system should be incubated for 1 hour at 37°C after addition of the haemolysin to the standardized cell suspension and is then ready for use. It has an O.D. of 0.200 which corresponds to a red cell concentration of 0.35×10^9 per ml.

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CHAPTER 11

HAEMAGGLUTINATION AND HAEMAGGLUTINATION-INHIBITION TESTS

E. KUWERT[1]

Until very recently rabies virus (RV) could not be determined quantitatively with reasonable accuracy except on the basis of its infectivity *in vivo*. When it became evident, however, that RV shares some of the characteristics of myxoviruses, especially the presence of surface projections morphologically similar to those on myxoviruses (Hummeler et al., 1967; Hummeler & Koprowski, 1969), it was reasonable to assume that haemagglutinin activity might occur and that a haemagglutination (HA) test could be developed (Habel, 1964).

The first report on haemagglutination of RV was published by Halonen et al. in 1968. The authors reported that the CVS strain agglutinated erythrocytes from geese, 2-day-old chicks, adult chickens, guineapigs, rats, sheep, and man (blood group O), provided the virus was grown in the absence of the HA-inhibitors present in every normal serum. Optimal conditions for demonstration of rabies haemagglutination included low temperature, pH 6.2, and the use of goose erythrocytes. Haemagglutination was not observed at room temperature or at 36°C. A positive haemagglutination pattern that has fully developed at 0–4°C rapidly reverts to negative at room temperature.

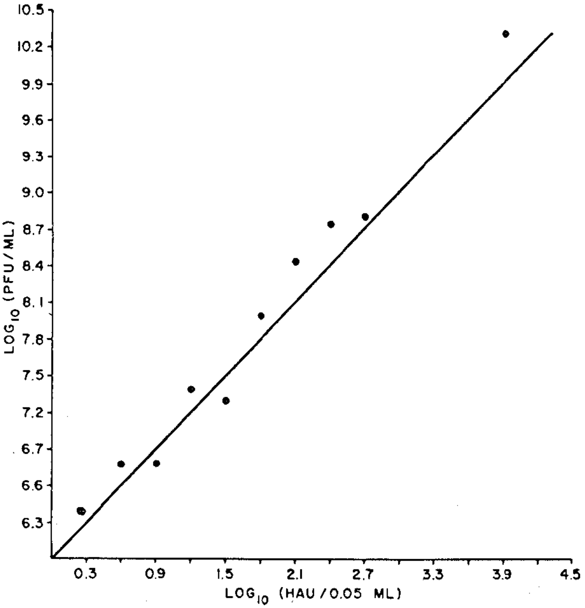
These observations could be confirmed and extended by Kuwert et al. (1968). Any strain of RV, regardless of the cell type in which it was propagated, will agglutinate goose erythrocytes under the conditions indicated above. RV grown in the CNS of laboratory animals, however, does not produce the haemagglutination phenomenon, even if partially purified. It is impossible to separate virus grown in brain from HA-inhibitors. RV propagated in tissue culture with bovine serum albumin as protein supplement can be quantitatively adsorbed on goose erythrocytes and eluted from them by raising the temperature and/or pH. Cells once treated with RV haemagglutinin retain their capacity to be agglutinated by the same or other strains of RV. Agglutination is not inhibited by pretreatment of the erythrocytes with potassium iodate or neuraminidase.

Results of sucrose gradient centrifugation of extracellular purified RV indicate that the HA-activity is due to the intact virions (Hummeler &

(1968). Treatment of gradient-purified RV with saponin (Schneider et al., 1971) disintegrates the virions into the nucleoprotein component and into non-infectious, haemagglutinating subunits of the virus envelope. The subunits derived from the coat (buoyant density 1.29 gm/ml) carry the total haemagglutinating and immunizing activity of the intact virus preparation. There is no evidence of a free "soluble" haemagglutinin. However, if the haemagglutinating envelope of RV is disintegrated by treatment with sodium desoxycholate, subunits exhibiting HA-inhibiting activity can be obtained, i.e., they have only one group capable of binding erythrocytes (Sokol et al., 1969).

The relationship between infectivity and HA-activity is linear, the ratio of plaque-forming units to HA-units being about 10^6 (Fig. 1). The HA-test can therefore be used in place of the more laborious infectivity titrations with good accuracy (mean quadratic deviation $\pm 0.3 \log_{10}$). However, this holds true only for virus released from the infected cells by "budding", but not for intracellular virus. The HA activity is destroyed by

FIG. 1. STRAIGHT-LINE RELATIONSHIP BETWEEN INFECTIVITY AND HA-ACTIVITY AS SHOWN BY PARALLEL TITRATIONS IN PLAQUE AND HA-TESTS



The points plotted are the mean values in the plaque test of 10 samples exhibiting the same HA endpoint. Standard deviation = $\pm$ 0.3 to $\pm$ 0.5 $\log_{10}$ PFU.
———————— = ratio of PFU to HAU = 10^6

proteolytic enzymes, compounds blocking sulfhydryl groups, and lipid solvents. RV-HA, therefore, is probably a lipoprotein that needs free SH-groups for the reaction with erythrocytes. Divalent cations, such as Ca^{++} and Mg^{++}, are not necessary for the HA reaction. There is no evidence that RV contains neuraminidase (Kuwert et al., 1968). The agglutination of goose erythrocytes by RV can be specifically inhibited by reacting the virus with homologous antibodies (haemagglutination inhibition (HI) test).

HA and HI tests can be performed by either a macro or a micro method. The macro method is performed in glass tubes using volumes between 0.25 ml and 0.5 ml of each reagent and 1.0-ml conventional serological pipettes for serial dilution of RV or its antibody. For the micro method, plastic microplates are used, with between 0.025 ml and 0.05 ml of each reagent, and microdiluters (see Fig. 2) or plastic microdroppers are used instead of glass pipettes. The endpoints obtained are similar, whichever method is adopted. Since, however, the micro technique offers so many advantages (saving of time, material, and laboratory personnel) and facilitates serological work tremendously (Sever, 1962) only this technique will be described in detail.

Haemagglutination Test for Detection of RV Haemagglutinin

Reagents

The preparation, sources, and storage of the reagents required are described in the annex on page 142.

Apparatus

Disposable Microtiter[1] U plates are preferred because they permit easier reading of the HA reaction. The test may be performed using either 0.025-ml or 0.05-ml antigen samples, the standard deviation being about the same ($\pm 0.3 \log_{10}$) for both quantities. Microtiter droppers or diluters are used for measuring the antigen and serological pipettes for transferring the candidate samples to the first row of cups of the microplates (dilution 1 : 2). The use of a multimicrodiluter (see Fig. 2) permits simultaneous titration of 12 samples, thus simplifying and speeding up the titration process. To ensure accurate measurement of volumes, occasional air bubbles within the "loops" can be eliminated by puncturing with a needle. For the same reason, the diluters should be carefully wetted before being used with BABS (bovine serum albumin borate saline, see Annex, page 142).

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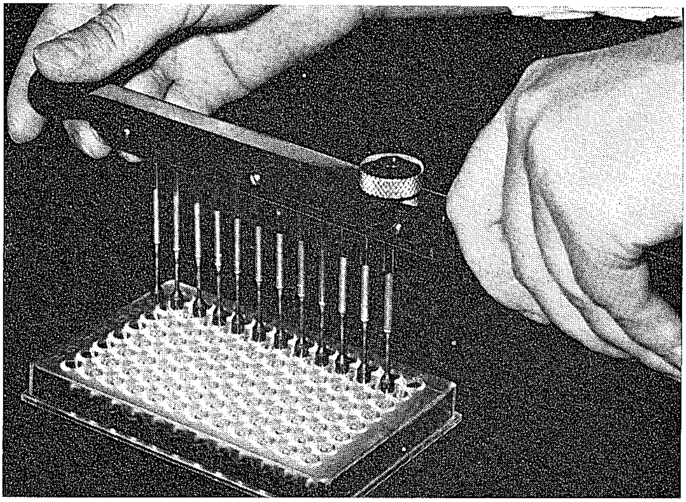
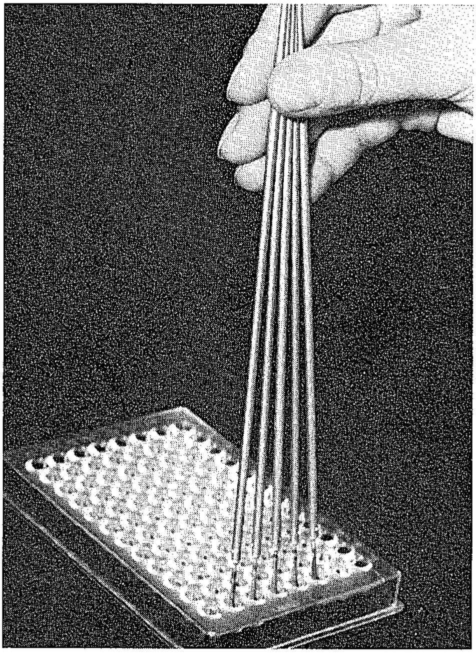
[1] The Microtiter kit is manufactured by Cooke Engineering Company, Medical Research Division, 735 North Saint Asaph Street, Alexandria, Va 22314, USA. Distributors include:

THE MULTIMICRODILUTER (0.05-ml CAPACITY) OR (*b*) 5 SAMPLES USING A BUNCH OF SIMPLE MICRODILUTERS (0.05-ml CAPACITY)



a)



b)

Procedure

It is important that all the reagents and the microplates be kept at 0–4°C throughout the operation. An ice-water slurry must be prepared for this purpose.

Calculate the number of cups of the microplate so that every candidate antigen and every standard (positive) and control (negative) antigen is titrated in duplicate.

1. Add 0.025 ml (0.05 ml) of ice-cold BABS, pH 9.0, to each of 8 wells of the U-microplate.

2. Add 0.025 ml (0.05 ml) of each antigen to the first cup in each of the two rows. This gives a 1 : 2 dilution of the antigen.

3. Transfer serially 0.025 ml (0.05 ml) by means of a simple microdiluter or a multimicrodiluter from the first and consecutive cup to each successive cup in the 2 rows. The dilutions of antigen obtained will range from 1 : 2 to 1 : 256.

4. Before addition of the goose erythrocytes place the microplate for 10 minutes on an ice-bath or in a refrigerator at 0°C in order to cool down the serially diluted antigen once more.

5. Add 0.025 ml (0.05 ml) of ice-cold 0.25 % goose erythrocyte suspension in phosphate-buffered saline (PBS), pH 6.4, to each cup including the controls.

6. Shake the plate and then seal with adhesive plastic tape and allow to incubate for 45 minutes at 0–4°C. This temperature must be controlled carefully. At 6–8°C the HA pattern develops only partially. Above 10°C no haemagglutination of goose erythrocytes by RV occurs.

7. Determine the pattern of HA. To improve reading the magnification mirror of the Microtiter kit should be used.

The HA pattern should be recorded as follows :

+ = complete agglutination : no sign of non-agglutinated cells.
± = partial agglutination : slight ring of non-agglutinated cells ; traces of agglutinated cells surround the button of non-agglutinated cells.
− = no agglutination : if the plate is kept tilted to one side, the cell sediment collects at the bottom of the cup in the form of a button.

Reading should be done within 2–5 minutes after transferring the plates from 0–4°C to room remperature. Elution of the virus from the erythrocytes is complete after 10 minutes at +22°C.

8. The HA endpoint is the highest dilution of the antigen giving partial haemagglutination. The reciprocal of this value is taken as 1 haemag-

Reagents

The preparation, sources, and storage of the reagents required are described in the annex on page 142.

Apparatus

The apparatus is essentially the same as that used for the haemagglutination test, with the addition of a refrigerated centrifuge.

Procedure

Reagents and items necessary for the first part of the HI test (reaction between RV and its specific antibody) and for adsorption of nonspecific inhibitors and heteroagglutinins from the sera are kept at room temperature. All reagents and items for the second step of the reaction (agglutination of erythrocytes by antibody-free RV) must be kept at 0–4°C during the whole operation. Plates containing the mixtures of RV and antiserum must be cooled on an ice-bath or in a refrigerator before addition of the erythrocyte suspension.

1. Inactivate each serum sample by heating for 30 minutes at 56°C.

2. Dilute each serum 1 : 10 in a 15-ml conical centrifuge tube with borate saline, pH 9.0 (0.1 ml of serum plus 0.9 ml of borate saline).

3. Add an equal volume (1 ml) of 25% kaolin in borate saline, pH 9.0.

4. Incubate at room temperature for 20 minutes with vigorous shaking every 5–10 minutes.

5. Centrifuge at 1000 *g* for 30 minutes. The centrifuge should be kept at a temperature of approximately 20°C. Do not remove the supernatant fluid.

6. Add 0.7 ml of 50% suspension of goose erythrocytes in PBS, pH 7.0.

7. Incubate for 30 minutes in an ice-bath, agitating every 10–15 minutes.

8. Centrifuge in a refrigerated centrifuge at 600 *g* for 10 minutes.

9. Remove and save the supernatant fluid. This represents a 1 : 20 dilution of the serum. If the serum specimen contains duck antibodies (vaccination with virus grown in duck embryos), steps 5, 7 and 8 should be repeated.

10. Add 0.025 ml of BABS to 2 rows of 12 cups each, omitting the first and last 3 cups of each row.

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[1] Based mainly on the recommendations of P. E. Halonen, personal communication, 1969.

11. Add 0.05 ml of treated serum with a 0.1-ml or 0.2-ml pipette to the first cup in each row for each serum and 0.025 ml to the last cup (serum control) in each row for each serum. This represents a 1 : 20 dilution of the serum.

12. Using a 0.025-ml diluter prepare serial twofold dilutions from 1 : 20 to 1 : 5120.

13. Add 0.025 ml of antigen containing 4 HA units to the first 9 cups of each serum.

14. Add 0.025 ml of BABS to the last cup of each serum. This represents the serum control.

15. Set up a back titration of the antigen to determine that the correct number of HA units have been used :

(*a*) Add 0.025 ml of antigen to the first and second of 5 cups in 2 rows (duplicate titration).

(*b*) Add 0.025 ml of BABS to the second, third, fourth and fifth cups.

(*c*) Prepare serial twofold dilutions starting with the second cup and using a 0.025-ml loop. These dilutions correspond to 4, 2, 1, 0.5 and 0.25 HA units per 0.025 ml.

(*d*) Add 0.025 ml of BABS to each cup.

16. Set up an erythrocyte control well containing 0.025 ml of BABS.

17. Incubate for 2 hours at room temperature and then cool the plates on an ice-bath or in a refrigerator (0°C) for 10–15 minutes.

18. Add 0.05 ml of a cold 0.25% suspension of goose erythrocytes in PBS, pH 6.4.

19. Seal the plate with adhesive plastic tape and incubate for 45 minutes on an ice-bath or in a refrigerator (0°C).

20. Read the back titration to determine the number of HA units used.

21. Read the erythrocyte control to confirm absence of agglutination.

22. Read the serum controls to confirm absence of nonspecific agglutination.

23. Read the titre of serum dilution that inhibits agglutination. The titre is the last dilution of serum causing partial inhibition of agglutination.

Annex

PREPARATION, SOURCES, AND STORAGE OF REAGENTS

Reagents Required for the Haemagglutination Test

Rabies virus suspension for titration of haemagglutinin

Only RV grown in tissue culture with BSA as protein supplement is suitable for HA titration. The HA activity of RV grown in the presence of complete serum (calf, horse, human, dog), or grown in CNS tissue *in vivo*, is inhibited by lipids or lipoproteins, probably α-lipoproteins. The virus preparation must have a concentration exceeding 10^6 units of infection (PFU or LD_{50}) per ml, otherwise no positive result will be observed. The virus sample can be preserved in any kind of medium or buffer with physiological pH (Eagle's medium, Hanks' or Earle's solution, PBS, etc.), provided it does not contain HA inhibitors.

Rabies virus working antigen

For the present, it is recommended that working antigen should be prepared from HEP Flury or Pitman Moore strains, grown in BHK 21 cells in the presence of 0.2–0.4% BSA. The antigen should contain at least 320 haemagglutinating units (HAU) per ml (= titre 1 : 16 per 0.05 ml), which corresponds to log infectivity values of 7.2 ± 0.3 PFU or LD_{50}. The standard antigen is distributed by the WHO International Reference Centre for Rabies at The Wistar Institute of Anatomy and Biology, Philadelphia, PA., USA.

Bovine serum albumin borate saline (BABS), pH 9.0

For the HA test both the candidate antigen and the working antigens (frozen suspension or rehydrated lyophilized stock) are serially diluted (dilution factor 2.0) in cold (+4°C) BABS (0.12 M sodium chloride solution + 0.05 M boric acid containing 0.4% of BSA), pH 9.

Stock solution

1.5 M sodium chloride

sodium chloride		87.6 g
distilled water	to make	1000.00 ml

0.5 M boric acid

boric acid		30.92 g
hot distilled water	to make	700.00 ml

When all the boric acid has dissolved, cool and dilute with distilled water to 1000.00 ml.

Borate saline, pH 9.0

1.5 M sodium chloride		80.00 ml
0.5 M boric acid		100.00 ml
1.0 N sodium hydroxide		24.00 ml
distilled water	to make	1000.00 ml

Check the pH in a pH meter

Bovine serum albumin borate saline (BABS), pH 9.0, 4% bovine albumin

bovine albumin, fraction V[1]	4.00 g
borate saline, pH 9.0	90.00 ml

Adjust the pH to 9.0 by addition of sodium hydroxide and then dilute with borate saline, pH 9.0, to 100.00 ml.

Sterilize using a Millipore filter (type G.S. ; pore size 0.22 µm) to avoid growth of contaminants on long storage. Store under refrigeration.

Working solution (prepared weekly)

4% bovine albumin borate saline, pH 9.0	10.00 ml
borate saline, pH 9.0	90.00 ml

Store under refrigeration.

Alsever's solution

dextrose		20.50 g
sodium citrate		8.00 g
citric acid		0.55 g
sodium chloride		4.20 g
distilled water	to make	1000.00 ml

Autoclave at 0.6 atm (6N/cm^2) for 10 minutes. Sterilization can also be performed using a Millipore filter (type G.S. ; pore size 0.22 µm).

Phosphate-buffered saline (PBS), pH 7.0

Solution A

sodium chloride	8.0 g
potassium chloride	0.2 g
calcium chloride, dihydrate	0.132 g
magnesium chloride, hexahydrate	0.1 g
distilled water	800.00 ml

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disodium hydrogen phosphate		1.15 g
potassium dihydrogen phosphate		0.2 g
distilled water		200.00 ml

1. Dissolve the salts in distilled water in the order listed.
2. Autoclave solutions A and B separately at a pressure of 10 N/cm^2 (1 atm) for 15 minutes.
3. Mix solutions A and B when cool, pouring B into A. Stir *slowly*. The final pH should be 7.0.
4. Dispense in quantitites of 500–1000 ml in sterile bottles.
5. Culture 1–5 ml in thioglycolate broth for a sterility test.
6. Store in the refrigerator.

Dextrose-gelatine-barbital buffer (DGB)

barbital		0.58 g
gelatine		0.60 g
barbital sodium		0.38 g
calcium chloride, anhydrous		0.02 g
magnesium sulfate, heptahydrate		0.12 g
sodium chloride		8.50 g
dextrose		10.00 g
distilled water	to make	1000.00 ml

The barbital and gelatine are dissolved in 250 ml of distilled water by heating. The other reagents are added and the solution is sterilized by filtration through a Millipore filter type G.S., pore size 0.22 µm. Store at 4°C. DGB may be used as an alternative to PBS.

Phosphate-buffered saline (PBS), pH 6.4

Stock solutions

1.5 M sodium chloride		
sodium chloride		87.67 g
distilled water	to make	1000.00 ml
2.0 M dibasic sodium phosphate		
disodium hydrogen phosphate, anhydrous		283.96 g
distilled water	to make	1000.00 ml
2.0 M monobasic sodium phosphate		
sodium dihydrogen phosphate, monohydrate		276.02 g
distilled water	to make	1000.00 ml

Virus adjusting diluent (VAD)

Solution A

1.5 M sodium chloride	100.00 ml
2.0 M disodium hydrogen phosphate	100.00 ml
distilled water	800.00 ml

Solution B

1.5 M sodium chloride	100.00 ml
2.0 M sodium dihydrogen phosphate, monohydrate . . .	100.00 ml
distilled water	800.00 ml

To obtain a final pH of 6.4, mix 32.0 ml of solution A with 68.0 ml of solution B. The pH should be checked potentiometrically.

Goose blood

Blood is obtained from the wing vein of an adult goose. There are no differences in HA sensitivity between erythrocytes obtained from different animals. After withdrawal the blood is immediately diluted with Alsever's solution (2 volumes of blood to 1 volume of Alsever's solution). Since goose erythrocytes are exceptionally resistant, the blood may be preserved in Alsever's solution for as long as 12 weeks at 4°C.

Goose erythrocytes in PBS

The volume of erythrocytes needed for 1–2 weeks of tests is washed 3–4 times in PBS or DGB and centrifuged at 500 *g* for 10 minutes. The last centrifugation is preferably made in a 12-ml conical, graduated centrifuge tube. The volume of packed cells is determined and a 10% stock suspension is made in PBS or DGB. Cells stored for a few days may be more sensitive to RV-HA than fresh cells.

The 0.25% suspension is prepared daily from the 10% stock suspension by adding 1.0 ml of the suspension to 39.0 ml of PBS, pH 6.4, in a conical flask. To ensure accuracy and reliability, smaller volumes should not be used. The erythrocyte suspension may be standardized photometrically.

Additional Reagents Required for the Haemagglutination-Inhibition Test

Serum samples

Before use in the HI test, serum samples should be inactivated by heating at 56°C for 30 minutes and non-specific HA inhibitors (probably α-lipoprotein) should be removed by adsorption on kaolin (tentative procedure). The samples should also be incubated with untreated goose cells to adsorb heteroagglutinins against this cell type. The adsorption procedures are described on page 140. For epidemiological surveys, one serum sample per individual animal is sufficient. For immunity studies after vaccination and for diagnostic purposes, it is preferable that two or more sera from each

Acid-washed kaolin[1] for adsorption of non-specific inhibitors is prepared as a 25% suspension in borate saline, pH 9 (Spence, 1960).

Goose erythrocytes, 50% suspension

For adsorption of heteroagglutinins against goose erythrocytes, a 50% erythrocyte suspension in PBS, pH 7.0, or DGB must be prepared.

All the other reagents required for the HI test are identical with those used in the HA test.

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[1] Obtainable from Amend & Co., New York, USA.

CHAPTER 12

PASSIVE HAEMAGGLUTINATION TEST FOR RABIES ANTIBODIES

R. E. DIERKS[1] *& PATRICIA M. GOUGH*[1]

The passive haemagglutination test is a new test for the measurement of rabies antibodies that has some distinct advantages over other tests at present in use. Once a satisfactory antigen has been prepared the test can be completed in several hours, thus giving a rapid indication of immunological response. As it uses a micro system it is also a relatively inexpensive test to perform. In the authors' laboratories this test has proved very useful for the titration of rabies antibodies in human sera, but until it has also been used successfully in other laboratories it cannot be accepted as a standard procedure. Descriptions of viral propagation, antigen preparation, and the test procedure are given below, with details of the problems encountered.

Virus Production and Concentration

Rabies virus CVS-11 (Kissling, 1958) is used for antigen production. This virus has been passaged 112 times in primary hamster kidney cell cultures and 13 times in baby hamster kidney cell cultures BHK-21 clone 13 (MacPherson & Stoker, 1962) and BHK-21 clone 13 S (Vaheri et al., 1965).

BHK-21 clone 13 cells are used for antigen production. They are grown in roller bottles (654.5 cm^2 surface area) in BHK 21 medium (MacPherson & Stocker, 1962) containing 10% (v/v) of heat-inactivated fetal calf sera, 10% (v/v) of tryptose phosphate broth, and antibiotics. A 48–72 hour stock culture of BHK-21 clone 13 cells is trypsinized, washed, and seeded on 100 ml of the medium, which is split among 6 flasks. A stream of carbon dioxide is passed through the bottles for 10 seconds to enhance cell attachment and the culture is then incubated at 37°C for 48 hours. The growth medium is removed and the monolayers washed twice with phosphate-buffered saline (Dulbecco & Vogt, 1954). Each roller bottle is infected with 10 ml of a 10^{-3} dilution of CVS-11 virus containing $10^{7.3}$ intracerebral MLD_{50}/ml. Diethylaminoethyl (DEAE) dextran, 50 µg/ml, is added

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is removed after absorption for 1 hour at 34°C and replaced by 100 ml of BHK-21 medium containing 10 ml of tryptose phosphate broth, 0.4 g of bovine serum albumin, and antibiotics. The roller cultures are then incubated for 4 days at 34°C with adjustment of the pH as needed to maintain it at 7.2 to 7.4. The tissue culture fluids are harvested, centrifuged for 20 minutes at 1000 *g* to remove cellular debris, and either concentrated or frozen at −70°C for future use. Each lot of virus is routinely tested for possible mycoplasma contamination and assayed for virus titre at this point. A minimum titre of $10^{6.5}$ MLD_{50} as assayed by intracerebral inoculation of 3-week old mice should be obtained if the lot is to be used for antigen production.

The virus is concentrated by the zinc acetate precipitation method (see also chapter 15, page 166). Fifty parts of infected tissue culture fluid are mixed with one part of 1.0 M zinc acetate solution (pH 5.0). The mixture is allowed to stand at 4°C for 20–30 minutes with occasional agitation. It is then centrifuged at 1000 *g* for 20 minutes and the supernatant fluid discarded. The precipitate is resuspended to $^{1}/_{20}{}^{th}$ of the original volume in a saturated solution of disodium EDTA adjusted to pH 7.8 by the addition of solid tris-(hydroxymethyl)-aminomethane. The resulting virus suspension is clarified by centrifugation at 1000 *g* for 20 minutes, dialysed against a 0.86% sodium chloride solution for 18 hours, and then stored at −70°C in small aliquots for future use. The precipitation procedure should give a virus recovery of 90–100% based on infectivity titrations.

Antigen Preparation

The concentrated rabies virus is coupled to goose erythrocytes for use in the test system. The erythrocytes are collected in modified Alsever's solution (Kent et al., 1946) and aged for at least 4 days before use. The red blood cells are washed 3 times in 0.86% sodium chloride solution immediately before use. The last discarded supernatant fluid should be clear and free of haemolysis. A complex is then formed between rabies virus and the goose erythrocytes with hydrated chromium chloride in a manner similar to that described by Gold & Fudenberg (1967), using equal volumes of virus suspension, 0.1% chromium chloride solution in 0.86% sodium chloride solution, and packed saline-washed erythrocytes. The virus is mixed with the packed red cells before addition of the chromium chloride coupling agent. This results in a better antigen then when the reagents are added together in any other order. After 5 minutes of interaction at room temperature the sensitized erythrocytes are washed 3 times in saline and suspended at a concentration of 0.25% in 0.86% sodium chloride solution for use in the test. The antigen complex is made up as a fresh reagent each day.

It has been found that the chromium chloride solution used as the coupling agent can be stored at 4°C for 1–2 weeks without any apparent adverse effects even though the colour spectrum shifts from dark green to grey green. If the concentration of chromium chloride is greater than 0.1% increased problems are experienced with agglutination of the red blood cells during the coupling process. The antigen cannot be used if agglutination occurs during its preparation.

The substitution of other commonly used buffers for 0.86% sodium chloride solution may result in the precipitation of salts accompanied by haemolysis of the erythrocytes.

Red blood cells from other species have also been used successfully in the test but goose cells have given the highest and most reproducible titres.

Passive Hemagglutination Test Procedure

The sera to be tested are heat inactivated at 56°C for 30 minutes and then adsorbed on an equal volume of 1% washed normal goose erythrocytes. After 30 minutes' incubation at 37°C the red blood cells are sedimented from the sera by centrifugation at 1000 *g* for 10 minutes. Twofold serial dilutions of the sera are made in a diluent composed of 0.4% bovine serum albumin, 0.05 M sodium borate, and 0.86% sodium chloride solution at pH 9.0 The sera are placed in V-shaped Microtiter plates[1] and the diluent added in volumes of 0.025 ml from microdiluters. Sensitized erythrocytes (0.05 ml) are added to each well and the plates are sealed with Cellophane tape and held at 4°C for approximately 1 hour before examination for haemagglutination. A sharply defined pellet of cells in the bottom of the well indicates a negative test. Positive reactions appear as a ring of cells on the sides of the wells or clumps of agglutinated cells.

Heat inactivation of the sera and adsorption on unsensitized erythrocytes are necessary to prevent the apparently nonspecific haemagglutination that occurs with some sera at low dilutions.

If it is more convenient for the dilutions to be made one day and the reactions to be observed on the following day (for example, when large numbers of sera are being titrated) the plates may be stored overnight at refrigerator temperatures without affecting the titres.

The passive haemagglutination test is a rapid, easily performed test and gives titres that correlate well with those obtained by serum neutralization tests on late postvaccination human sera. The haemagglutination test was performed on 347 sera with serum neutralization titres of 1 : 5 to 1 : 3000 obtained from subjects who had received pre-exposure vaccination or post-exposure prophylaxis. Only 8 gave haemagglutination titres that

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dilution. For measurement of the early immune response (specific anti-rabies macroglobulins or IgM antibodies) the passive haemagglutination test is more sensitive than the serum neutralization test (Gough & Dierks, 1971).

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CHAPTER 13

GEL-DIFFUSION TECHNIQUES

P. LÉPINE [1]

Immunoprecipitation reactions are among the most sensitive tests for the identification of specific antigen and specific antiboby. Studies by Oudin (1946) and by Ouchterlony (1949) on gel-diffusion techniques have led to important applications of immunoprecipitation reactions in the diagnosis of virus diseases. Several papers have been published on the use of these techniques in rabies: with a proper appreciation of the sensitivity of these methods as well as their limitations it is possible, under certain conditions, to make a rapid identification of a rabies virus or a rabies immune serum.

It is necessary, however, that the antigen taking part in the reaction should contain sufficient virus and that the antibody should be present in sufficient quantity if the reaction is to yield a visible precipitate. From this it follows that:

(*a*) *If unknown antibodies are to be identified* (for example, for the diagnosis of latent rabies in dogs in areas where rabies is enzootic), it is necessary to have available a sufficiently concentrated rabies antigen to produce a reaction. In such cases, therefore, the unknown serum is reacted either with an antigen prepared from the brains of suckling mice inoculated with a very virulent strain of high-titre fixed virus or, as described by Grasset & Atanasiu (1961), with a concentrated rabies antigen prepared by tissue culture.

(*b*) *If an unknown virus is to be identified,* it is necessary to have available a hyperimmune serum prepared by immunizing the donor animal with a rabies virus obtained from an animal of a different species from the one under investigation. This situation frequently arises when dogs suspected of rabies have been killed and their brains submitted for examination, either to confirm a diagnosis of rabies reached on the basis of a search for Negri bodies or to provide additional evidence when the histological examination has yielded doubtful or negative results. For this type of test, successful use has been made both of the Pasteur Institute serum made by hyperimmunization of horses with rabbit brain (Villemot & Provost, 1958a, b; Thiéry,

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serum made by immunization of donkeys with the Flury strain of avianized virus (Villemot & Provost, 1958 a, b).

Methods and Material

Petri dish method

This method was developed by Villemot & Provost (1958 a, b), who were the first to show the presence of two precipitable fractions in tissues containing rabies virus. The technique consists in punching out small cups in an agar gel poured in a Petri dish and then filling some of these with rabid brain tissue and the remainder with immune serum. Soluble antigen and precipitating antibody diffuse towards one another through the gel and react to give a precipitate, which forms two lines clearly visible to the naked eye.

Medium

Bacto-Agar	15 g
methyl orange	0.03 g
thiomersal	0.2 g
water	1000 ml

The methyl orange facilitates the reading of the results and the thiomersal inhibits bacterial growth.

The medium is dispensed in 30-ml portions in 22-mm tubes and stored at 4°C. When required, the tubes are placed in the water-bath and the melted medium poured into scrupulously clean Petri dishes. After cooling, the medium should be perfectly homogeneous and clear, and should be free from all particulate matter. Of the stains that have been tried, methyl orange seems to have given the best results, while 0.012% Congo red has also given good readings. According to Lazear et al. (1958), 0.03% methylene blue is preferable if black-and-white photographs are to be taken.

Cups

Using as a punch a piece of glass tubing 7 mm in internal diameter and 8 mm in external diameter, remove 7 small vertical cylinders of the solidified medium so as to leave 6 cup-shaped holes spaced 5 mm apart around the circumference of a circle 14 mm in radius, with the seventh hole at the centre. (These dimensions were fixed after having determined the distance between the antibody source and the antigen source that gave the best differentiation of the precipitation lines.) A drop of the molten medium is then pipetted into each cup so as to ensure that the bottom is sealed. This is necessary to stop any leakage of serum, which would mix with the antigen and prevent the formation of precipitation lines.

Antigen

For the identification of sera, we use as antigen either brains of dogs infected with a highly virulent strain of street virus or brains of young rabbits inoculated with the 1955th passage of the Pasteur fixed virus strain.

Antibody

We use either purified horse serum produced by the Pasteur Institute or crude serum from donkeys immunized by a series of injections of HEP Flury virus followed by a series of injections of LEP Flury virus, immunity being maintained by monthly injections of 24 ml of a 33% suspension of chicken embryo infected with an LEP Flury strain.

Test procedure

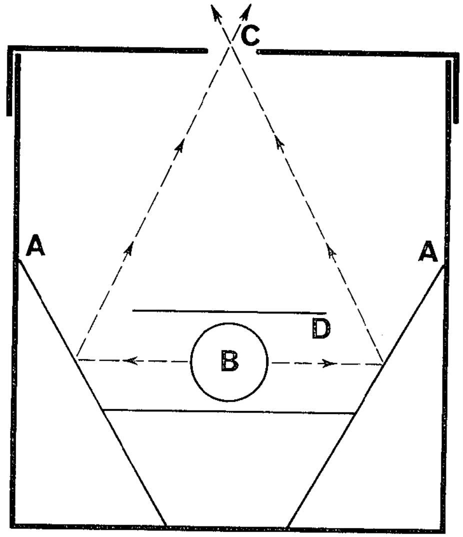
Two techniques can be used. The first consists in placing rabid brain tissue and normal brain tissue in alternative peripheral cups and running antirabies serum into the central cup ; the normal tissue serves as a control. The second technique is the reverse of the first, i.e., the peripheral cups are filled alternately with immune serum and normal serum, while rabid brain tissue is placed in the central cup.

Reading the results

The Petri dishes are placed in an incubator at 37°C and the cups refilled with serum at intervals of about 12 hours, or as often as necessary to prevent them becoming empty. Readings are taken after 24, 48 and 72 hours, depending on the time of appearance of the precipitation lines. They are facilitated by the use of dark-ground illumination. This may be provided by means of a simplified version of the device described by Lazear et al. (1958) : a plywood box with detachable lid is lined with tin foil and two plane mirrors are so arranged inside that light from a 100-watt lamp placed midway between them is reflected through a circular hole, 8 cm in diameter, cut in the lid. A screen covered with black paper placed between the lamp and the hole prevents the direct light from reaching the eye, so that the readings are made against a dark ground (see Fig. 1).

Optimum distance between antigen and antibody cups

This should be determined using a known antigen, which is placed in two divergent rows of cups, while a central row, containing the same



Modified from Lazear et al. (1958)

A. Plane mirrors
B. 100-watt lamp
C. 8-cm hole in detachable lid of box lined with tin foil
D. Screen covered with black paper

According to experiments made by Villemot & Provost, the precipitation lines are most easily and rapidly observed using cups 7 mm in diameter placed 7 mm apart.

Interpretation of results

It is necessary to make sure that suspensions of normal animal brain do not give a precipitate when tested against the serum. If normal rabbit brains are tested against serum produced by immunization with rabbit brain, a precipitation line appears after 48 hours and a second line, fainter than the first and nearer to the antigen cup, appears after 72 hours. These lines are due to the presence in the serum of anti-rabbit-brain antigen ; if the antigen is first removed by mixing the serum in a mechanical shaker with ground normal rabbit brain and then recovering the serum by centrifugation, the lines do not appear.

If the brain of a rabid dog is used, two lines appear after 48 hours. These curve very slightly away from the antigen cups. They are attributed to two antigen fractions, one with a molecular weight approximately equal to that of the antibody, the other with a molecular weight slightly higher.

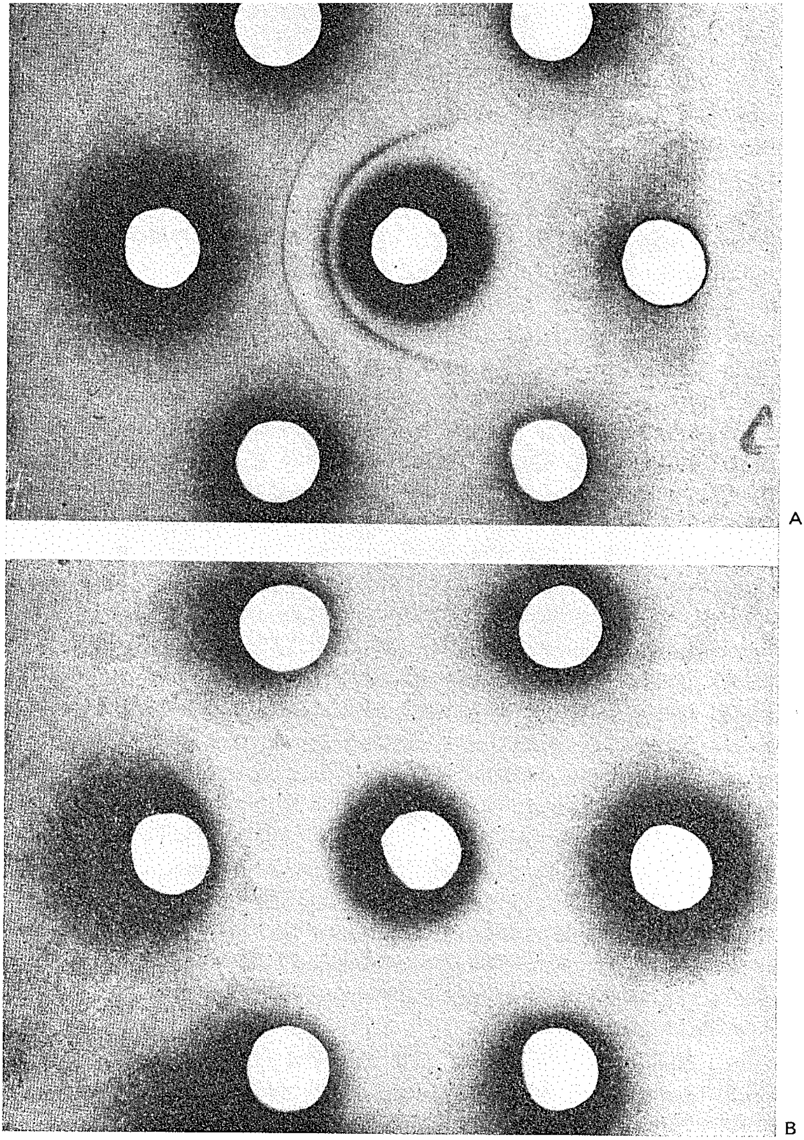
Microprecipitation on slides

This method was first described by Scheidegger in 1955 and later adapted to the study of poliovirus by Grasset et al. (1958). A modification suitable for use with tissue cultures of rabies antigen was developed by Grasset & Atanasiu (1961).

The method of preparing the microscope slides is similar to that used by Grasset et al. A 2% solution of agar is diluted immediately before use with an equal volume of barbital buffer of pH 8.2. Next, 3 ml of the melted agar are poured on to histological slides and allowed to solidify. By means of a special tool, a fixed pattern of 6 cups disposed about a central cup and 5 mm distant from it is punched in the agar. Each cup is 2 mm in diameter and has a capacity of approximately 0.004 ml (in some cases, cups 5–7 mm in diameter with a larger capacity have been used). Concentrated immune serum is placed in the central cup. The peripheral cups are filled alternately either with tissue culture of the rabies antigen and control tissue culture or with concentrated brain suspensions from animals suspected of rabies and from control animals ; the samples should be taken from Ammon's horn, the hippocampal gyrus, or the basal optic ganglion (see chapter 5, page 62). The slides are then left at laboratory temperature in an airtight vessel containing an atmosphere saturated with water vapour. The precipitation lines begin to appear after 12 hours. After 40–72 hours, the slides are dried and stained with amidoschwartz solution.

Slides prepared in the same way may also be used for electrophoretic analysis. As in the technique described above, a barbital buffer of pH 8.2 is used ; the brain suspension or antigen tissue culture is placed in the central cup and an electric current is passed through the gel. Two lateral grooves are then made in the agar, one of which is filled with antirabies immune serum and the other with normal horse serum. Precipitation is allowed to take place under the same conditions as before. When the reaction is positive, a double precipitation line is again seen, extending from the specific serum to the cup containing the antigen (Fig. 2). Under certain conditions, this technique has proved sensitive for quite low titres of virus in the brain (10^{-2}).

Gel diffusion is a technique that calls for careful execution but gives accurate results. What is its practical value ? Villemot & Provost (1958 a, b) have employed it on a routine basis for the examination of brains sent to their laboratory. Their material comprised 44 brains from dogs, 16 from cats, 2 from monkeys and 2 from human patients, all of whom were suspected of rabies. The test gave a positive result with 24 canine brains and one human brain. These findings were in 100% agreement with the results of animal inoculation experiments, but they were obtained in 48 hours whereas animal experiments take much longer.



Reproduced, by permission, from Grasset & Atanasiu (1961)

A. Double precipitation line resulting from reaction of rabies antigen with specific antirabies serum. The thicker, principal line is situated near the central cup containing the rabies antigen. The thinner, secondary line is situated half-way between the central cup and the three cups on the left containing the antirabies serum. No line is visible between the central cup and the three cups on the right, which contain normal horse serum.

B. Control slide. No lines are visible on either side of the central cup containing the control antigen (tissue-culture preparation). The three cups on the left contain antirabies serum, the three on the right normal horse serum.

Magnification ×4.

Atanasiu et al. (1963) used the technique of Grasset & Atanasiu (1961) to identify rabies virus cultured on BHK-S13 cells in the presence of hyperimmune horse serum from the Pasteur Institute. The results were excellent.

The gel-diffusion technique is thus both specific and sensitive. However, it has its limitations. Thiéry (1960) has made a comparison between the method of Villemot & Provost and the classical methods for the routine identification of rabies virus. He concludes that when brain tissue is examined by the gel-diffusion method, the result will be positive if sufficient rabies antigen is present; when serum is examined, a positive result is obtained if there is an increase in the α_2 globulins, an increase that is normally associated with rabies but varies in degree from one animal to another. From this it follows that, while a positive result is unequivocal evidence of rabies, a negative result may be due to the absence from the antigen or the serum of the specific fractions characteristic of rabies or to a disproportion between the quantities of antigen and antibody entering into the reaction. Grasset & Atanasiu likewise found that the immunoprecipitation reaction gave consistent results only if antigen and antibody were present in suitable proportions; they obtained negative results not only with tissue cultures or brain tissues having a low virus titre although giving a positive inoculation test, but also with sera of low neutralizing power.

It must therefore be concluded that although the gel-diffusion technique is accurate and sensitive it cannot at present be considered entirely reliable. A positive result permits a rapid diagnosis of rabies or the presence of rabies antibody. On the other hand, a negative result does not necessarily exclude rabies or the presence of rabies antibody.

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ELECTRON MICROSCOPY

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The electron microscope is essentially a research tool and only rarely will it be necessary to employ it for diagnostic purposes in preference to the light microscope. It is of practical value in the examination of vaccine strains for the detection of contaminants not readily recognized by other means. In rabies research, the electron microscope has played a key role in elucidating the structure and the morphogenesis of the virus in experimental animals and in tissue cultures (Atanasiu et al., 1963 ; Miyamoto & Matsumoto, 1965).

Structural Studies

In longitudinal section and in negative contrast, the complete virus particle resembles a bullet (Fig. 1). It measures 180×75 nm. A fringe of surface spikes can be seen and the surface itself appears as a dense mass with occasional particles exhibiting a distinct surface arrangement as hexagons forming a honeycomb. The spikes are about 6.0–7.0 nm long (Hummeler et al., 1967).

The internal nucleoprotein exists in helical form (Fig. 2). When isolated from disrupted virions, it appears as a single-stranded right-handed helix 1 µm in length and 16 nm in diameter, with a periodicity of 7.5 nm. The helical structure of the nucleocapsid is extremely labile. The uncoiled strand of the helix is about 4.2 µm long with a varying width of 2–6 nm depending on the angle of viewing (Sokol et al., 1969).

Studies of Morphogenesis

Experimental animals

Two virus strains were used: the Pasteur strain passed in rabbit brain and a wild strain isolated from the brain of a fox. Suckling mice 4–6 days old were inoculated intracerebrally. The incubation period for fixed rabies virus was 4–6 days and for street rabies virus 10–20 days. The animals were killed when they showed signs of imminent death.

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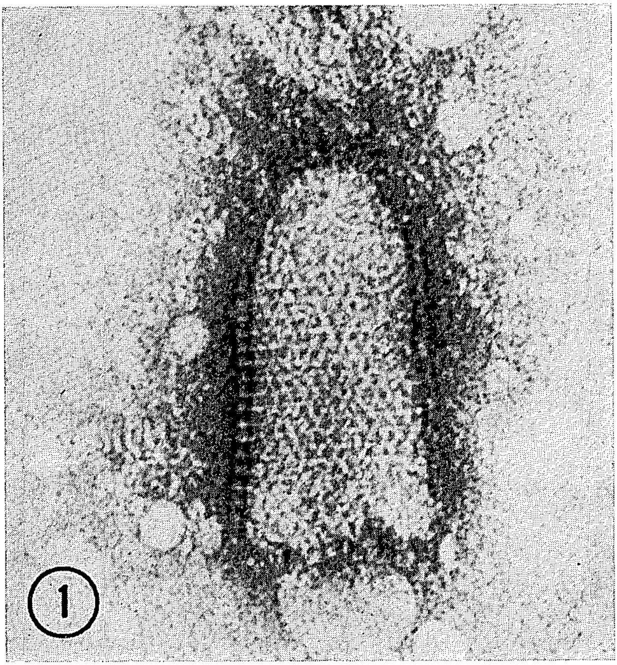
The Ammon's horn from each side of the brain was removed in the cold. One part was kept for direct examination, after emulsification in phosphotungstic acid, and the other part was used for light microscopy of inclusion bodies and thin sectioning.

FIG. 1. RABIES VIRION IN NEGATIVE CONTRAST



× 260 000

Fragments of the Ammon's horn were fixed for 2 hours at 4°C in 4% glutaraldehyde (pH 7.2). The fragments were cut into small cubes and then fixed for 18 hours in isotonic osmic acid (1% solution, pH 7.2). After dehydration, the cubes were embedded in a mixture of Epon [1] and Araldite.[2] Thin sections were stained for 60 minutes in a 5% solution of uranyl acetate and for 20 seconds in lead citrate and then examined under the electron microscope.

Virions were rarely seen in negatively stained emulsions of Ammon's horn cells. These virions often possessed a double membrane, projections, and transverse striations. Occasionally the nucleocapsids extruded from

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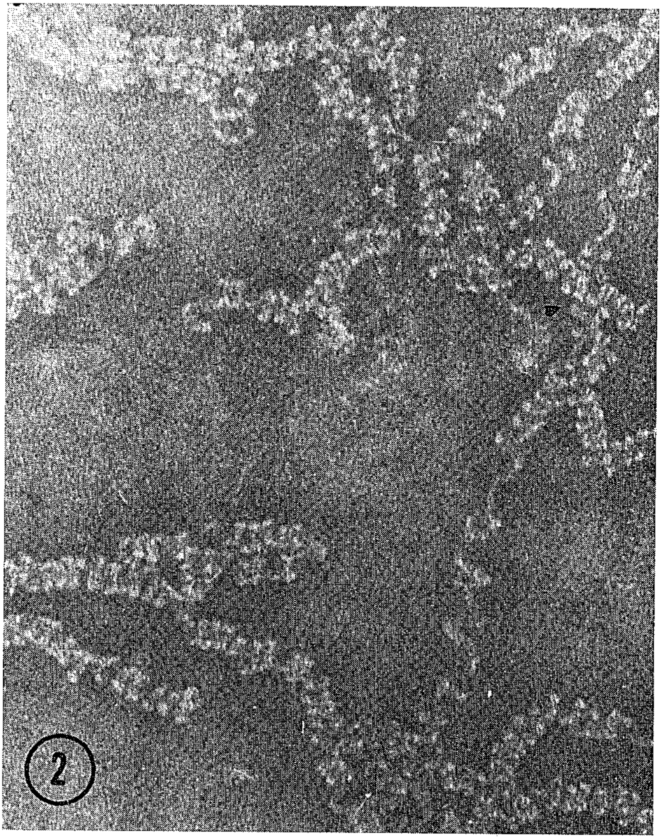

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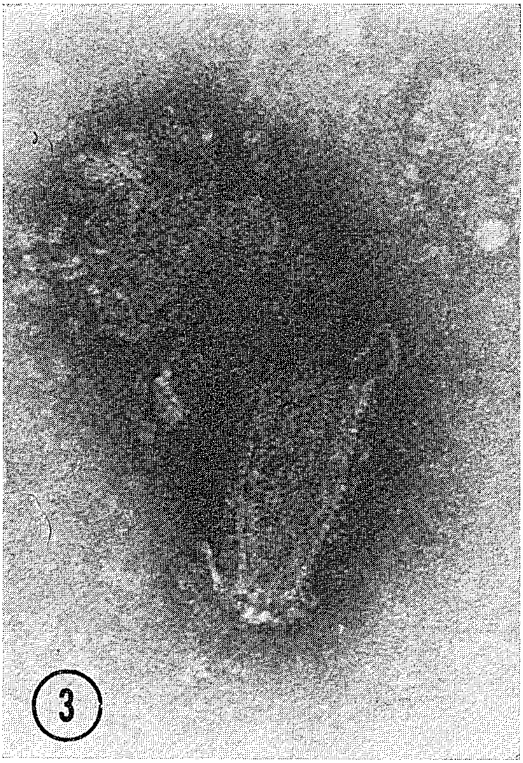
the caudal part of the virion (Fig. 3). The size and shape of these virions were identical with those demonstrated in Fig. 1, derived from tissue cultures.

In thin sections it has been possible to observe the evolution of the infection. This seems to differ considerably depending on the virus strain used. Fixed virus caused appearance of specific inclusions, or matrices, in the pyramidal cells of the Ammon's horn. The fine structure of these inclusions was homogeneous, consisting of thin filaments (3.0–5.0 nm). Virions were found only at the periphery of the inclusions, where they formed on membranes of the endoplasmic reticulum and ergastoplasmic vesicles. These virions can be seen in transverse and longitudinal sections in Fig. 4.

Street virus showed this kind of morphogenesis only rarely. The morphology of the matrix tended to be heterogeneous. Besides the charac-

FIG. 3. RABIES VIRION AFTER EMULSIFICATION OF AMMON'S HORN PYRAMIDAL CELLS (NEGATIVE STAINING)



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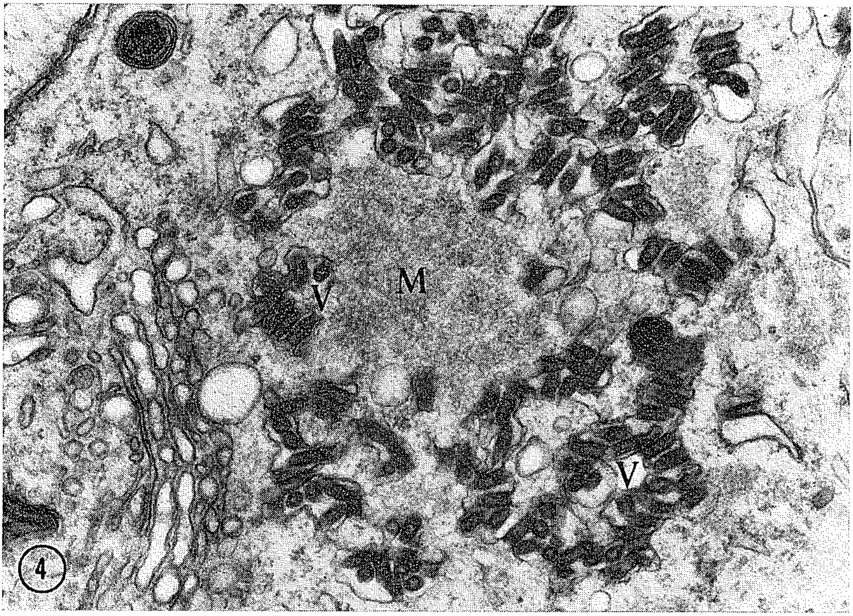
teristic filaments—presumably the nucleoprotein—considerable condensation of the material in the matrix was observed. The density increased with time and membranous structures appeared. They were 25–30 nm in diameter, frequently exhibiting a transverse striation. Distinct double membranes without any relation to pre-existing cell membrane structures also emerged. These double membranes often aligned in pairs and the gap between them (50–60 nm) corresponded to the internal diameter of a complete virion (Fig. 5).

It is evident that in the morphogenesis of street virus in the Ammon's horn of the mouse not only replication of the nucleocapsid strands but also the formation of the envelope and the assembly of the virus particle take place within the matrix (Atanasiu et al., 1969).

Tissue culture studies

The fixed rabies virus strains CVS and Flury HEP were used in these

INFECTION. THE STRUCTURE OF THE MATRIX IS HOMOGENEOUS. VIRIONS FORM ON PRE-EXISTING CELL MEMBRANES



× 50 000

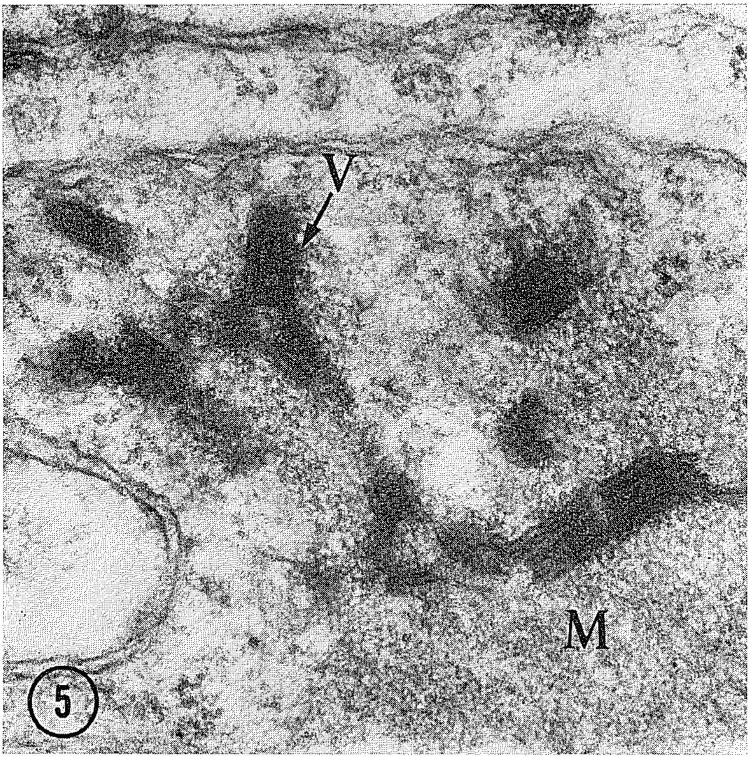
Monolayers of baby hamster kidney cells (BHK-21) were maintained in Basal Medium (Eagle) (see Chapter 9, Annex 2) in Hanks's solution supplemented with 10% fetal calf serum.

After removal of the medium, the cell monolayers were washed and exposed for 1 hour at 37°C to the virus at an input multiplicity of 5–10. After virus adsorption the cells were washed, maintenance medium was added, and the cultures were incubated at 34°C to be harvested at predetermined intervals.

The harvested cells were washed and fixed in 3% glutaraldehyde for 1 hour. Subsequently they were fixed in osmic acid (1%, pH 7.2), dehydrated, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined in an electron microscope.

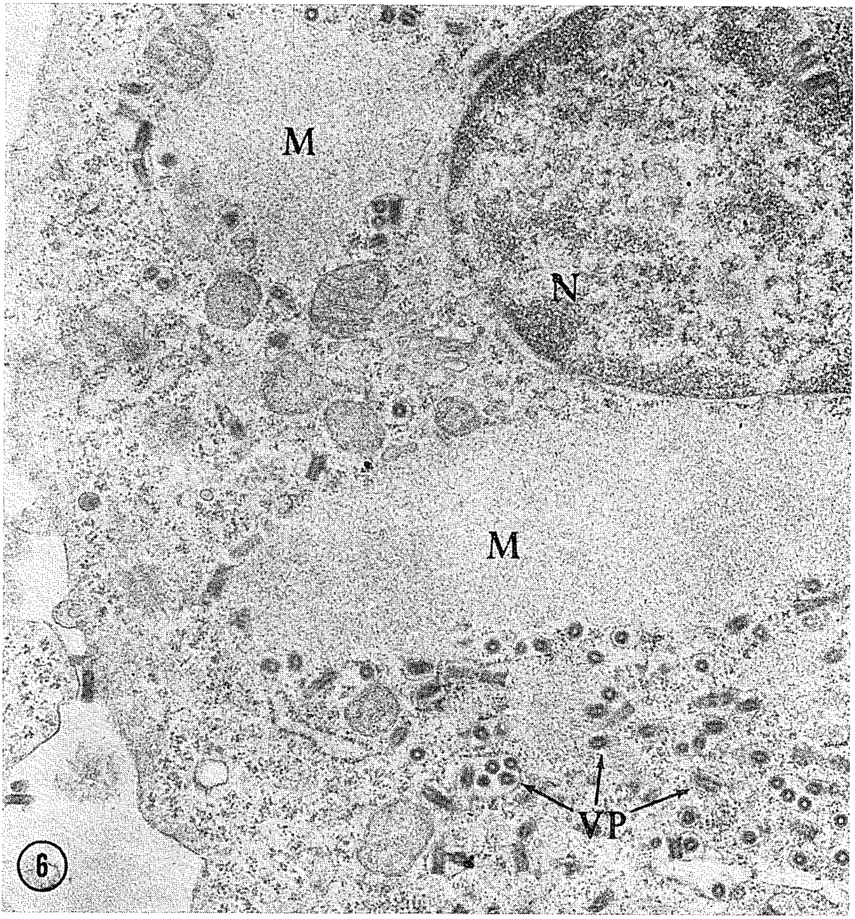
In tissue cultures, the first structural changes become obvious 6–7 hours after infection. These changes consist in the appearance of granular material, which in time becomes filamentous and replaces cell organelles. Virus particles later appear in or around these matrices (Fig. 6). This morphological evidence of virus replication in the cytoplasm is identical with the inclusions seen in neurones in the brain of infected animals. The filamen-

FIG. 5. STREET VIRUS: THIN SECTIONS OF PYRAMIDAL CELLS OF THE AMMON'S HORN. DOUBLE MEMBRANES AND COMPLETE VIRIONS CAN BE FOUND IN THE MATRIX



× 80 000

tous strands are present in the cytoplasm in helical form, similar to those in the virus particles, and thus constitute the nucleoprotein of the virus. During virus maturation on pre-existing cell membranes, the nucleoprotein strands are incorporated into the virions (Hummeler et al., 1968). With some attenuated virus strains, however, a different morphogenesis can be observed. Virus particles emerge on the periphery of the matrices and inside them without obvious involvement of pre-existing cell membranes. Whether these differences in morphogenesis, which are also evident in brain cells of experimental animals, reflect differences in biological characteristics or whether they represent different degrees of attenuation to a given cell is impossible to answer at present (Hummeler & Koprowski, 1969). The experimental results described in this chapter show that all the distinct features of the morphogenesis of rabies viruses seen in neurones



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M = matrix VP = virus particles N = nucleus

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CHAPTER 15

PURIFICATION OF RABIES VIRUS AND ISOLATION OF ITS COMPONENTS *

F. SOKOL [1]

In this chapter, only methods that have proved effective for the concentration and purification of rabies virus and those used for the isolation of some viral components are described. Procedures suitable only for partial purification of cell-associated virus (Cox et al., 1947 ; Muller, 1950 ; Tagay et al., 1953 ; Sawai et al., 1954 ; Thomas et al., 1965 ; Turner & Kaplan, 1967) are not included. Procedures for the complete fractionation of disrupted rabies virions into pure antigens have not yet been worked out ; however, methods for the isolation of pure viral nucleocapsid and pure viral ribonucleic acid (RNA) have recently been elaborated and are described in this chapter.

Source of Virus

The purification procedure outlined below is effective when infectious tissue culture fluid is used as a source of rabies virus. Infection with rabies virus causes only a weak cytopathic effect, if any, in susceptible cells ; there is, therefore, relatively little contamination of extracellular virus with cellular components. The virus synthesized in the cells is rapidly released into the medium. Thus, at the time of harvest, at least half of the infectious virus contained in the culture can be recovered from the culture fluid (Sokol et al., 1968). So that binding of serum components to the virions and contamination of purified virus preparations with aggregated serum components will not occur, the virus should be grown in cell cultures supplemented with media in which serum has been replaced by a 0.1–0.3% solution of bovine serum albumin (BSA). The virus can be propagated in monolayer cultures of any susceptible cell, such as baby hamster kidney (BHK), African green monkey, human diploid, or chick embryo cells. The highest yields of fixed rabies virus have been obtained in BHK-21 cells. Optimum conditions for propagation of the virus in this cell line are described in Chapter 9.

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only partially effective in the purification of cell-associated virus (Sokol et al., 1968).

Concentration of Rabies Virus

Depending on the strain of fixed rabies virus and the tissue culture system used, infectious tissue culture fluids contain 1×10^7 to 5×10^8 plaque-forming units (PFU) of virus, 4–128 haemagglutination units (HAU), and 8–256 complement-fixing units (CFU) per ml. As the first step in the purification procedure, the virus must be concentrated and freed of the bulk of contaminants of low molecular and high molecular weight. This can be accomplished by any of the methods suitable for virus concentration, provided the temperature is kept low (0–10°C), the pH of the suspension is kept within the range 6.8–8.0, and extreme ionic strengths (strongly hypertonic or strongly hypotonic solutions) are avoided. Three methods have been successfully used at The Wistar Institute for concentration and partial purification of extracellular rabies virus from crude preparations that have been freed of cells and cell debris by low-speed centrifugation : precipitation of the virus by zinc acetate, sedimentation of the virions by high-speed centrifugation, and ultrafiltration through nitrocellulose membranes. The precipitation and sedimentation methods are useful for the concentration of small volumes, their advantage being separation of the bulk of the nonvirion proteins from the virus particles. The ultrafiltration method is suitable for rapid concentration of large volumes of infectious tissue culture fluid, particularly when a battery of filters is employed.

Precipitation of rabies virus by zinc acetate

Rabies virions are precipitated by Zn^{+2} ions when 50 parts of the infectious tissue culture fluid (pH 7.4–7.8) are mixed with 1 part of 1 M zinc acetate (pH 5.0). After mixing, the pH of the virus suspension drops to about 6.8. The mixture is allowed to stand at 0–4°C for 20–60 minutes and the precipitate formed is collected by centrifugation at 1000 *g* for 40 minutes. The clear, essentially virus-free, supernatant fluid is discarded and the sediment is suspended in a saturated solution of the disodium salt of ethylenediaminetetraacetic acid (EDTA) (about 11.7 g/100 ml of water) adjusted to pH 7.8 by the addition of solid tris(hydroxymethyl)aminomethane (Tris). The volume of the EDTA solution used for suspending the virus must be at least 1.25% of that of the infectious tissue culture fluid. The resulting virus suspension must be clear ; residual precipitate, if present, can be dissolved by an additional amount of EDTA solution. The preparation of concentrated virus is then clarified by centrifugation at 1000 *g* for 20 minutes. It contains almost all the infectious virus present in the tissue culture fluid, variable amounts of BSA, and small amounts of cellular components and compounds of low molecular weight trapped in the precipitate.

Sedimentation of rabies virus by high-speed centrifugation

The sedimentation coefficient of infectious rabies virions is about 600 S (Neurath et al., 1966). To sediment a few milligrams of virus from a relatively pure, concentrated preparation, we found it useful to perform the high-speed centrifugation in a swinging bucket rotor, because under these conditions the sediment is collected in the centre of the bottom of the tube. When the virus suspension is subjected to high-speed centrifugation in an angle rotor, the sediment is spread on the wall of the tube. The transparent, opalescent, virus pellet, unlike the sedimented cellular fragments, can be readily suspended in buffer solution, even without sonic treatment or homogenization. On the other hand, when the virus is sedimented from crude preparations, such as serum-containing infectious tissue culture fluid or homogenate of infected cells, the virus-containing pellet has to be resuspended by sonic treatment for 1 or 2 minutes at 10 kHz and 0°C. In the presence of protein carrier, the infectivity of the virus is not affected by this treatment.

The routine procedure adopted in our laboratory for the sedimentation of rabies virus from preparations of extracellular virions grown in tissue culture in the presence of BSA, is as follows : 90 ml (three 30-ml portions) of clarified virus suspension is centrifuged in the SW 25.1 swinging bucket rotor of a Spinco centrifuge at 50 000 *g* (22 000 rev/min) for 60 minutes at 4°C. The supernatant fluid is carefully removed by suction and the sediment is overlaid with a small volume of a solution of 0.13 M sodium chloride, 0.05 M Tris chloride and 0.001 M EDTA (NTE buffer, pH 7.8). The contents of the tubes are kept for 3–4 hours at 4°C and rocked occasionally. Gentle pipetting with a Pasteur pipette is then sufficient to resuspend the virus. Cell debris and aggregated virions, which cannot be easily resuspended under these conditions, are removed by centrifugation at 1000 *g* for 20 minutes at 4°C. Almost all the infectivity present in the original preparation is recovered in the resuspended sediment.

Concentration of rabies virions by ultrafiltration

Infectious tissue culture fluids can also be concentrated by ultrafiltration through a nitrocellulose membrane of 0.01-μm to 0.015-μm porosity. Nonvirion proteins that are present in the preparation, such as BSA or serum proteins and the rabies-specific "soluble" antigens, are concentrated together with the virions by this procedure. The operations involved in a typical concentration experiment are as follows : For removal of cell debris porosity which has been prewashed with a solution of 0.13 M sodium chloride and 0.05 M Tris chloride, (NT buffer, pH 7.8), and a 1% solution of BSA in the same buffer. The filtration apparatus, equipped with the Sartorius SM12 133 filter,[1] is then sterilized by 2% formalin solution and washed with NT buffer. The prefiltered virus suspension is concentrated 50-fold to 100-fold by suction, maintaining a flow rate of about one ml per minute per cm^2. The virus-free filtrate is discarded and the concentrate, containing all the infectivity, is clarified by low-speed centrifugation.

Purification of the Virus

The procedure, successfully used for purification of extracellular rabies virus grown in tissue culture with BSA-supplemented medium (Sokol et al., 1968), is shown in Fig. 1. Concentration of the virus by precipitation with zinc acetate can be replaced by sedimentation or ultrafiltration without affecting the yield of purified virus or the purity of the final preparation. Filtration of the concentrated virus through the Sephadex G-75 column separates the impurities of low molecular weight and a part of the BSA from the virions. The volume of the virus suspension to be filtered should not exceed 10% of the column volume. The virus preparation is of a high degree of purity even before the final banding of the virus in a sucrose density gradient (Fig. 2). Banding of the virus in a sucrose density gradient is always accompanied by a twofold to threefold decrease in infectivity, because many of the virus particles are disrupted during removal of the sucrose by dialysis. To minimize inactivation, the purified virus has to be dialysed stepwise against sucrose solutions of decreasing concentration.

From 25% to 50% of the infectivity contained in the infectious tissue culture fluid is recovered in the final preparation. Some properties of the purified rabies virus are summarized in Table 1. The purity of the virus

TABLE 1. PROPERTIES OF PURIFIED RABIES VIRUS (STRAIN HEP FLURY)

Specific infectivity [a]	10^{10} PFU/mg protein
Specific HA activity [a]	10^4 HAU/mg protein
Specific CF activity [a]	5×10^3 CFU/mg protein
Buoyant density in sucrose solution at 20°C	1.17 g/cm^3
Sedimentation coefficient	550–650 S
Antibody types induced after immunization	virus neutralizing, HAI[b], CF, and lytic[c], and precipitating

[a] The specific biological activities of the virus were determined as described by Sokol et al. (1968).

[b] HAI = haemagglutination inhibition.

[c] The properties of lytic antibodies have been described by Wiktor et al. (1968).

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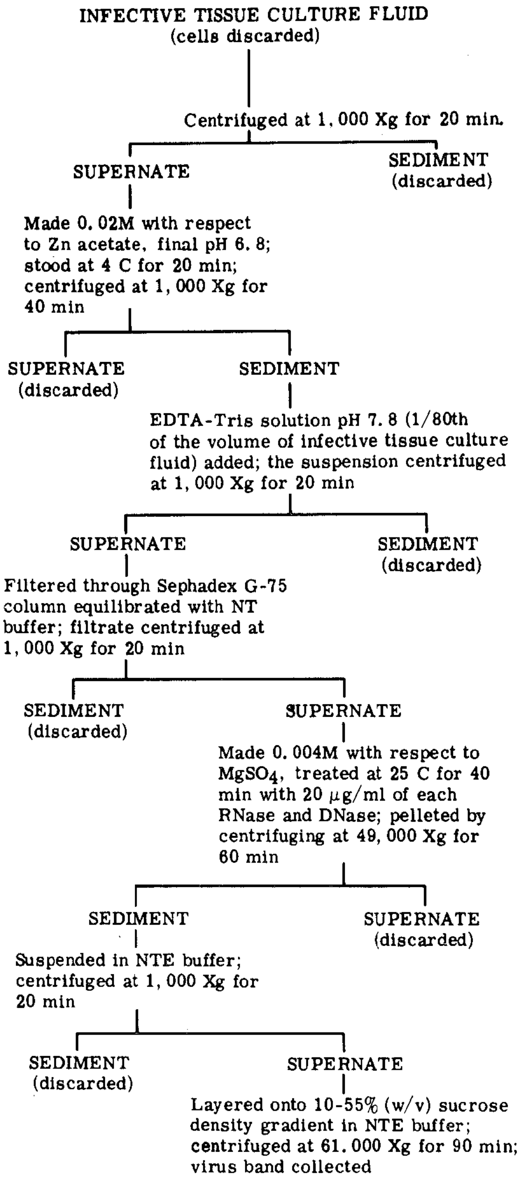
[1] Obtainable from Dr Fritz Sartorius GmbH, Göttingen, Federal Republic of Germany.

FIG. 1. PURIFICATION OF EXTRACELLULAR RABIES VIRUS, GROWN IN CELL CULTURE, IN MEDIUM CONTAINING BSA IN PLACE OF SERUM


INFECTIVE TISSUE CULTURE FLUID (cells discarded)
Centrifuged at 1,000 Xg for 20 min.
SUPERNATE
SEDIMENT (discarded)
Made 0.02M with respect to Zn acetate, final pH 6.8; stood at 4 C for 20 min; centrifuged at 1,000 Xg for 40 min
SUPERNATE (discarded)
SEDIMENT
EDTA-Tris solution pH 7.8 (1/80th of the volume of infective tissue culture fluid) added; the suspension centrifuged at 1,000 Xg for 20 min
SUPERNATE
SEDIMENT (discarded)
Filtered through Sephadex G-75 column equilibrated with NT buffer; filtrate centrifuged at 1,000 Xg for 20 min
SEDIMENT (discarded)
SUPERNATE
Made 0.004M with respect to $MgSO_4$, treated at 25 C for 40 min with 20 μg/ml of each RNase and DNase; pelleted by centrifuging at 49,000 Xg for 60 min
SEDIMENT
SUPERNATE (discarded)
Suspended in NTE buffer; centrifuged at 1,000 Xg for 20 min
SEDIMENT (discarded)
SUPERNATE
Layered onto 10-55% (w/v) sucrose density gradient in NTE buffer; centrifuged at 61.000 Xg for 90 min; virus band collected

RNase = ribonuclease; DNase = deoxyribonuclease.

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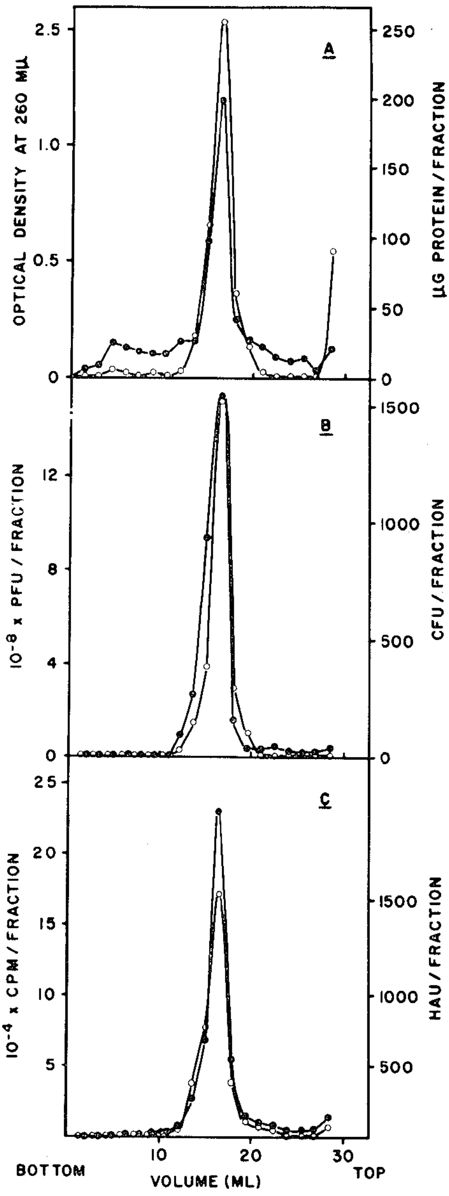
AND PARTIALLY PURIFIED RABIES VIRUS (STRAIN HEP FLURY)



0.5 ml of ^{3}H-uridine-labelled virus, concentrated by precipitation with zinc acetate and filtered through a Sephadex column, was layered on 28 ml of a 10–60% (w/v) linear gradient of sucrose in NTE buffer and centrifuged at 4°C in the SW 25.1 rotor of a Spinco centrifuge for 90 minutes at 61 000 *g* (24 500 rev/min). Fractions were then collected through a hole punctured in the bottom of the tube and analysed. The three parts of the figure represent the same centrifugation experiment. The virus was grown in BHK-21 cells in the presence of BSA.

A. ○ = protein content; ● = optical density at 260 nm.

B. ○ = CFU; ● = PFU.

C. ○ = HAU; ● = radioactivity of ^{3}H-uridine.

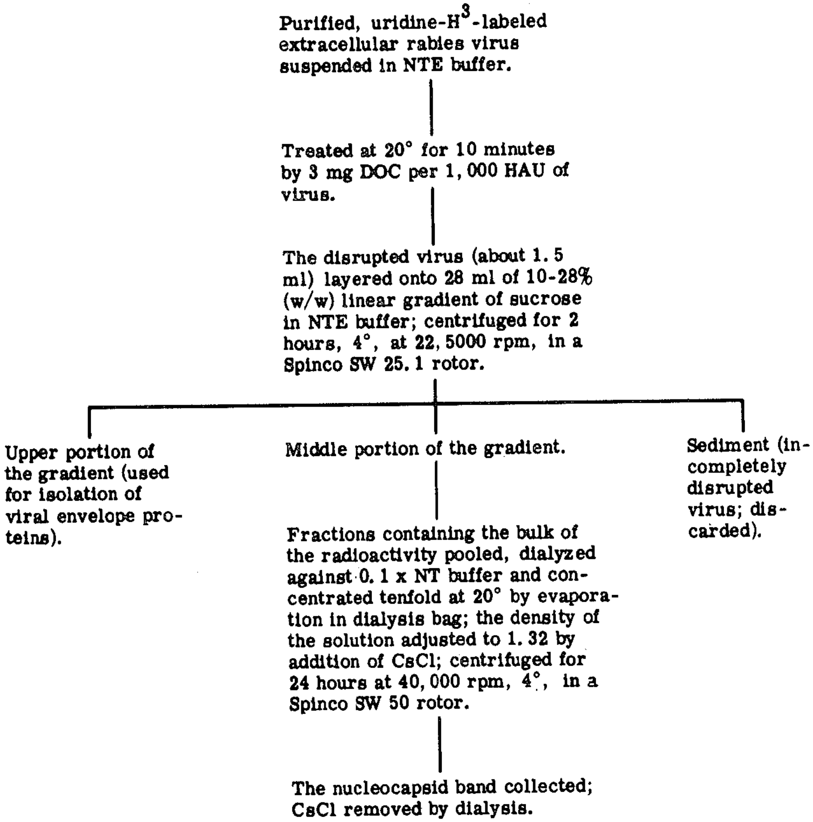
cpm = counts per minute.

Reproduced from Sokol et al. (1968) by permission of the American Society of Microbiology preparation is usually higher than 95% (Sokol et al., 1968). Viral preparations purified by the procedure outlined above contain, in addition to infectious, 180-nm long virions, a variable proportion of truncated virus particles (Sokol et al., 1968). In the absence of carrier protein, the infectivity of purified virus is extremely labile. After the addition of 0.5% to 1% of BSA, the virus van be stored at −70°C for several months without essential loss of infectivity.

Isolation of Virion Components

The morphology of the envelope and of the nucleocapsid of rabies virions is described in detail in Chapter 14. The envelope of rabies virus consists of proteins, lipids, and carbohydrates. The nucleocapsid, i.e., the core of the virus particle, contains 96% of protein and 4% of ribonucleic acid (RNA).

FIG. 3. PROCEDURE USED FOR DISRUPTION OF RABIES VIRIONS AND PURIFICATION OF THE RELEASED NUCLEOCAPSID



Purified, uridine-H^3-labeled extracellular rabies virus suspended in NTE buffer.

Treated at 20° for 10 minutes by 3 mg DOC per 1, 000 HAU of virus.

The disrupted virus (about 1. 5 ml) layered onto 28 ml of 10-28% (w/w) linear gradient of sucrose in NTE buffer; centrifuged for 2 hours, 4°, at 22, 5000 rpm, in a Spinco SW 25. 1 rotor.

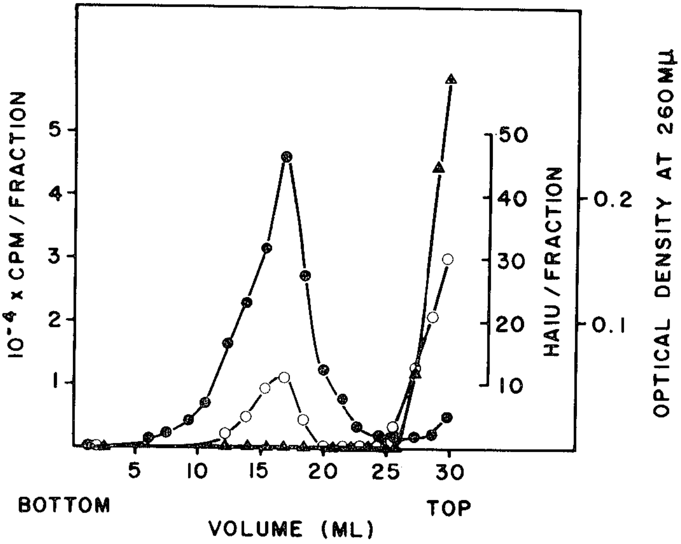
Upper portion of the gradient (used for isolation of viral envelope proteins).

Middle portion of the gradient.

Sediment (incompletely disrupted virus; discarded).

Fractions containing the bulk of the radioactivity pooled, dialyzed against 0. 1 x NT buffer and concentrated tenfold at 20° by evaporation in dialysis bag; the density of the solution adjusted to 1. 32 by addition of CsCl; centrifuged for 24 hours at 40, 000 rpm, 4°, in a Spinco SW 50 rotor.

The nucleocapsid band collected; CsCl removed by dialysis.



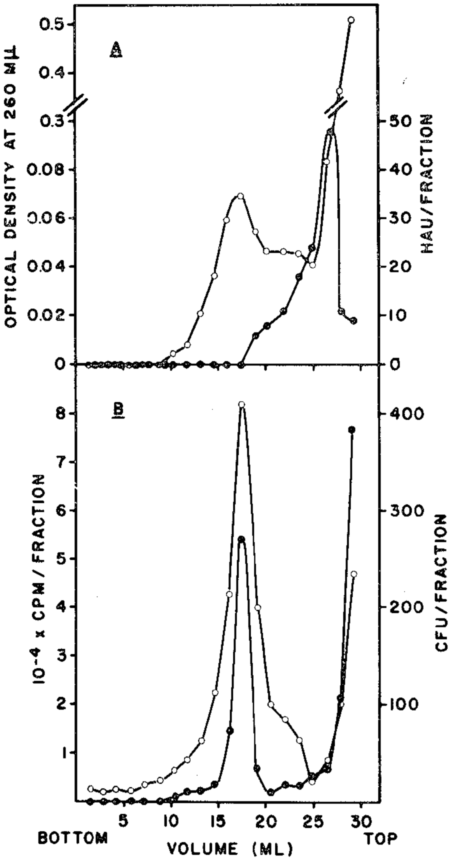
^{3}H-uridine-labelled, purified rabies viru (1.8×10^{4} HAU) suspended in 0.45 ml NTE buffer was disrupted by treatme with a 0.35-ml, 20% (w/v) DOC solution distilled water (4 mg DOC/1000 HAU) ar dialysed exhaustively at 4°C against NT buffer. The disrupted virus was centrifuge in a sucrose density gradient, as describe in Fig. 3. Fractions were collected an total counts (●——●), optical densi at 260 nm (○——○), and HAI activi (▲——▲) were determined.

Treatment with sodium deoxycholate (DOC) solubilizes the lipids, disintegrates the viral envelope, and releases the nucleocapsid (Sokol et al., 1969). So far only the viral RNA and the nucleocapsid have been isolated in a pure form from preparations of disrupted virus. The isolation of the mixture of disintegrated envelope components has also been described, but procedures for its fractionation have not been elaborated.

The separation of the nucleocapsid from envelope components is described in Fig. 3. Treatment with DOC does not affect the complement-fixing (CF) activity of the virus preparation, but destroys its infectivity and decreases its haemagglutinating (HA) activity about 1000-fold. The haemadsorbing ability of the coat component, derived from the intact viral haemagglutinin, is, however, not abolished. This component can be detected and titrated by making use of its inhibitory effect on the agglutination of goose erythrocytes by intact rabies virus. The distribution of haemagglutination inhibition (HAI) activity after fractionation of disrupted rabies virions by centrifugation in a sucrose density gradient is shown in Fig. 4. The HAI test is carried out in the following way:

Serial twofold dilutions of the envelope components of the virus are made in a solution of 0.12 M sodium chloride and 0.05 M borate (pH 9.0), containing 0.4% of BSA. An equal volume (0.05 ml) of suspension, containing 4 HAU of intact rabies virus, is then added to each well of the plastic plate, and the mixture is kept at 0°C for 30 minutes. An addition of 0.1 ml of 0.5% goose erythrocytes suspended in a solution of 0.15 M sodium chloride and 0.2 M phosphate (pH 6.4), is made to each dilution, the plates are incubated at 0°C for an additional hour, and the endpoint of the reaction is then read. The reciprocal of the highest initial dilution of the envelope components showing complete inhibition of haemagglutination is considered to represent the number of haemagglutination inhibition units (HAIU) per ml.

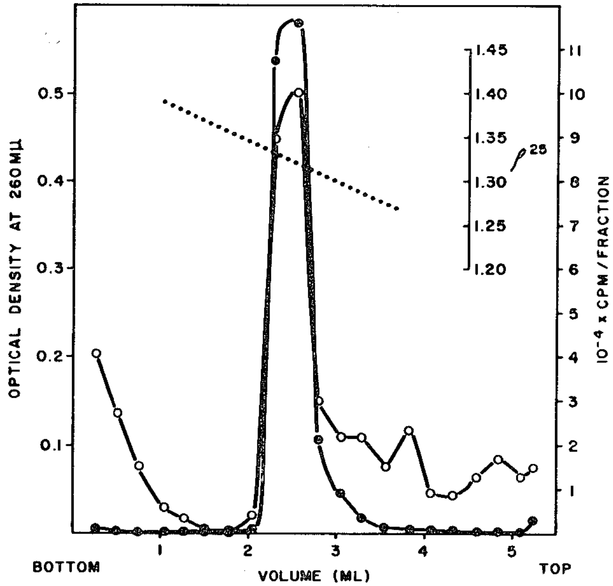
FIG. 5. FRACTIONATION OF THE CF AND HA ACTIVITY OF DOC-DISRUPTED RABIES VIRUS BY CENTRIFUGATION IN A SUCROSE DENSITY GRADIENT



A suspension of purified, ^{3}H-uridine-labelled rabies virus in 0.7 ml of NTE buffer, containing 3.5×10^{3} CFU and 1.0×10^{4} HAU, was treated with a 0.2-ml, 20% (w/v) DOC solution in distilled water (4 mg DOC/1000 HAU). The disrupted virus was centrifuged in sucrose density gradient as described in Fig. 3. Fractions were collected, and optical density at 260 nm (○———○, panel A), HA activity (●———●, panel A), total radioactivity (○———○, panel B), and CF activity (●———●, panel B) were determined. Specific antirabies rabbit serum was used for determination of the CF activity of the fractions. The slowly sedimenting and ^{3}H-uridine-labelled material is most likely cellular RNA contaminating the preparation.

tionation by rate zonal centrifugation is shown in Fig. 5. The nucleocapsid, recovered from the middle portion of the sucrose density gradient, bands after equilibrium centrifugation in a cesium chloride density gradient at 1.32 g/cm^3 density (Fig. 6). The properties of the nucleocapsid are summarized in Table 2.

FIG. 6. EQUILIBRIUM CENTRIFUGATION OF RABIES VIRUS NUCLEOCAPSID IN CESIUM CHLORIDE SOLUTION



^{3}H-uridine-labelled nucleocapsid in 4.5 ml of NT buffer, isolated from 2.1 × 10^6 HAU of rabies virus by treatment with DOC and subsequent centrifugation in sucrose density gradient, was mixed with 2 g of CsCl and the resulting solution was centrifuged for 25 hours at 150 000 *g* (40 000 rev/min) at 4°C in a Spinco SW50 rotor. Fractions were collected and the density (......) was determined. The total radioactivity (●———●) was determined and the optical density at 260 nm was read after dilution of the fractions with 0.2 ml of distilled water.

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Viral RNA can be isolated from purified virus or nucleocapsid preparations by the following procedure. A purified preparation of rabies virus or rabies virus nucleocapsid in NTE buffer is mixed with sodium dodecylsulfate (SDS) to yield a final concentration of 1% of SDS and shaken at 25°C for 10 minutes with an equal volume of 80% phenol containing 0.1% of 8-hydroxyquinoline. The aqueous phase is separated by low-speed centrifugation and treated once more with phenol. After the concentration of sodium chloride in the aqueous phase has been adjusted to 1 M, the RNA is precipitated by treatment with 2.5 volumes of ethanol at −20°C for 24 hours. The precipitate is then collected by low-speed centrifugation,

TABLE 2. PROPERTIES OF NUCLEOCAPSID ISOLATED FROM RABIES VIRUS (STRAIN HEP FLURY)

Chemical composition :	96% protein, 4% RNA
Morphology :	Single-stranded helix ; the helix is about 1-μm long, its diameter is 10 nm and it has a periodicity of about 7.5 nm ; the length of the uncoiled strand is about 4.2 μm and its width varies from 20 to 6.5 nm
Buoyant density in CsCl solution at 25°C :	1.32 g/cm^3
Sedimentation coefficient at 20°C :	200 S
Molecular weight :	1.2×10^8 amu [a]
Biological activity :	Noninfectious, exhibits CF activity, induces the formation of CF antibodies
Protein moiety of the nucleocapsid :	12 protein molecules, each with a molecular weight of 62 000 amu, per one turn of the helix ; i.e., about 1 850 protein molecules per nucleocapsid
RNA moiety of the nucleocapsid :	One single-stranded RNA molecule with a molecular weight of about 4.6×10^6 amu per nucleocapsid ; the RNA is noninfectious

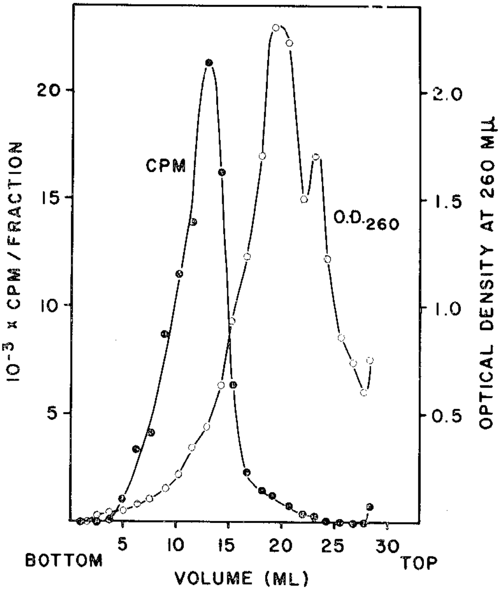
[a] amu = atomic mass unit.

washed 3 times with a 1 : 3 mixture of 1 M sodium chloride and ethanol, dried, and dissolved in NTE buffer. If necessary, the viral RNA can be further purified by rate zonal centrifugation in a sucrose density gradient (Fig. 7). The sedimentation coefficient of rabies virus RNA is 45 S. It can be recovered from the sucrose solution by precipitation with ethanol or freed of sucrose by dialysis.

Isolation of the Viral Nucleoprotein (Free Nucleocapsid) from Virus-Infected Cells

Cells infected with rabies virus contain, at a late stage of the infectious cycle, cytoplasmic inclusions composed of viral nucleoprotein, i.e., of free nucleocapsid particles that have not been incorporated into the virions (Hummeler et al., 1968). Through the use of a procedure similar to that described by Compans & Choppin (1967) for the isolation of cell-associated,

FROM VIRIONS IN A SUCROSE DENSITY GRADIENT



Viral RNA was isolated from purified, ^{3}H-uridine-labelled rabies virus preparation (1.1×10^4 HAU) by treatment with SDS and phenol and mixed with 1.26 mg of unlabelled BHK-cell RNA. The RNA was concentrated to 0.4 ml by ethanol precipitation and layered on 28 ml of a 5–20% (w/v) linear layered gradient of sucrose in NTE buffer. The gradient was centrifuged for 7 hours at 50 000 *g* (22 000 rev/min) 4°C, in a Spinco SW 25.1 rotor. Fractions were collected and the total radioactivity (●———●), as well as the optical density at 260 nm (○———○), were determined.

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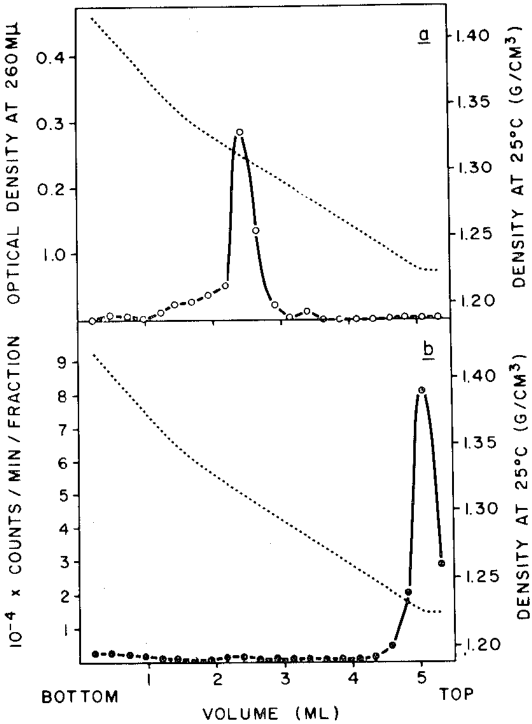
specific for rabies virus was recently isolated in a pure form from infected BHK cells (F. Sokol & S. Fein, unpublished experiments).

About 3×10^8 BHK cells, infected at an input multiplicity of 1 and harvested 48–72 hours after infection, are scraped off the glass or dispersed by treatment with trypsin, sedimented by low-speed centrifugation, washed twice with NT buffer, suspended in 10 ml of distilled water, and kept at 4°C for 1 hour. The suspension of disrupted cells in then clarified by centrifugation at 5000 *g* at 4°C for 20 minutes. The supernatant fluid is saved and the sediment is suspended in 1 ml of distilled water. The resuspended sediment is then homogenized by 4 strokes in a Dounce homogenizer and clarified by low-speed centrifugation as before. The sediment is discarded and the supernatant fluids are pooled. Magnesium sulfate is then added to the nucleocapsid-containing cell extract to make a final concentration of 5×10^{-3} M, and the suspension is treated with 20 μg of deoxyribonuclease per ml at 20°C for 30 minutes. Portions of 4.5 ml are then mixed with 2 g of cesium chloride and the resulting solution is centrifuged at 150 000 *g* (40 000 rev/min) at 4°C for 24 hours in the SW 50.1 rotor of a Spinco

FIG. 8. EQUILIBRIUM CENTRIGUFATION OF CELL-ASSOCIATED, FREE NUCLEOCAPSID IN CESIUM CHLORIDE SOLUTION



Nucleocapsid, derived from 3×10^8 BHK cells infected with the HEP Flury strain of rabies virus and prepurified by one banding in CsCl solution, was recentrifuged in CsCl solution in NT buffer of 1.30 g/cm³ average density for 24 hours at 150000 *g* (40 000 rev/min) at 4°C in the SW50 rotor of a Spinco centrifuge. Fractions were then collected through a hole punctured in the bottom of the tube and the density (......) was determined. The optical density at 260 nm (o———o) was read after dilution of the fractions with 0.2 ml of distillled water (panel *a*). In parallel, the same amount of uninfected cells, mixed with 2×10^4 HAU of ^{3}H-uridine-labelled intact virus, was disrupted and extracted as described in the text. After the first banding in CsCl solution, the density (......) and the radioactivity (●———●) of the fractions were determined (panel *b*).

centrifuge. The nucleocapsid band at 1.32 g/cm³ density, which can be also located by its turbidity, absorption at 260 nm, or the radioactivity of its RNA or protein component, is collected and freed from impurities of low molecular weight and cesium chloride by dialysis against NT buffer. The nucleocapsid is then rebanded in cesium chloride solution (Fig. 8, panel *a*). The rebanded nucleocapsid is again freed from cesium chloride by dialysis and clarified by low-speed centrifugation. Nucleocapsid particles isolated by this method are not derived from intracellular rabies virus, because nucleocapsid could not be obtained by a similar procedure from an artificial mixture of noninfected cells and intact rabies virions (Fig. 8, panel *b*). The properties of the cell-associated, free nucleocapsid are similar to those of

Since this chapter was written, there have been a few new developments in the isolation and characterization of rabies virus components. Rabies virus has been shown to contain 4 major and 1 minor protein component. The protein moiety of the viral envelope consists of a glycoprotein (GlP) and 2 membrane proteins (EP2 and EP3), whereas the viral RNA is surrounded by a major (NCP) and a minor (NCP-M) core protein component to form the viral nucleocapsid (Sokol et al., 1971). NCP was shown to be a phosphoprotein that can be converted into NCP-M by treatment with trypsin, which splits off the phosphorylated segment of this polypeptide (Sokol & Clark, 1973). The spikes emerging from the viral envelope consist of the viral glycoprotein and they can be solubilized, together with the viral lipids (glycolipids), by treatment of the virus with nonionic detergents (Nonident P-40 or tri-(*n*-butyl) phosphate and Tween 80) (György et al., 1971 ; Neurath et al., 1972). The viral " core particles ", from which the lipids and the envelope glycoprotein have been largely removed by this treatment, contain the viral nucleocapsid bound to the 2 membrane proteins. " Core particles " can be separated from the solubilized glycoprotein by velocity centrifugation in a sucrose density gradient. Crude glycoprotein preparations obtained in this way are still contaminated with small amounts of other viral proteins.

Recent developments in the purification of rabies virus are described in Chapter 17.

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CHAPTER 16

ALUMINIUM PHOSPHATE METHOD FOR RABIES VIRUS PURIFICATION

L. SCHNEIDER[1]

Methods suitable for the concentration and purification of rabies virus derived from tissue culture preparations have been described in Chapter 15. A further method that has recently been described consists in the batch adsorption of the virus on aluminium phosphate gel equilibrated with 0.07 M phosphate buffer at pH 7.1–7.3, followed by elution from the gel with 0.3 M phosphate buffer at pH 8.0 (Schneider et al., 1971).

The aluminium phosphate method is a rapid and relatively simple procedure that permits 200-fold to 300-fold concentration of the virus with average purification factors of about 2000, based on the activity/protein ratios of the original and the purified material.

Materials

Buffer solutions for adsorption and elution

Stock solution 1: 53.4 g of disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) are dissolved in 1000 ml of distilled water. This gives a 0.3 M solution.

Stock solution 2: 40.8 g of potassium dihydrogen phosphate (KH_2PO_4) are dissolved in 1000 ml of distilled water. This gives a 0.3 M solution.

Adsorption buffer: 1000 ml of stock solution 1 and 400 ml of stock solution 2 are mixed with 4600 ml of distilled water. This results in a 0.07 M solution with a pH of 7.1–7.3.

Elution buffer: 948 ml of stock solution 1 are mixed with 52 ml of stock solution 2. This results in a 0.3 M solution with a pH of 8.0.

Tris-EDTA-NaCl (TEN) buffer

60.75 g of Tris chloride, 1.86 g of ethylenediamine tetraacetic acid (EDTA), and 58.0 g of sodium chloride are dissolved in 1000 ml of distilled water. The pH is adjusted to 7.6 by addition of hydrochloric acid. For use this solution is diluted 10-fold with distilled water.

Tris-sucrose-stabilizer (TSS) buffer

6.057 g of Tris chloride, 2.033 g of magnesium chloride hexahydrate ($MgCl_2.6H_2O$), 1.470 g of calcium chloride dihydrate ($CaCl_2.\ 2H_2O$), of distilled water. The pH is adjusted to 7.6 by addition of hydrochloric acid. TSS buffer may be used as an alternative to TEN buffer.

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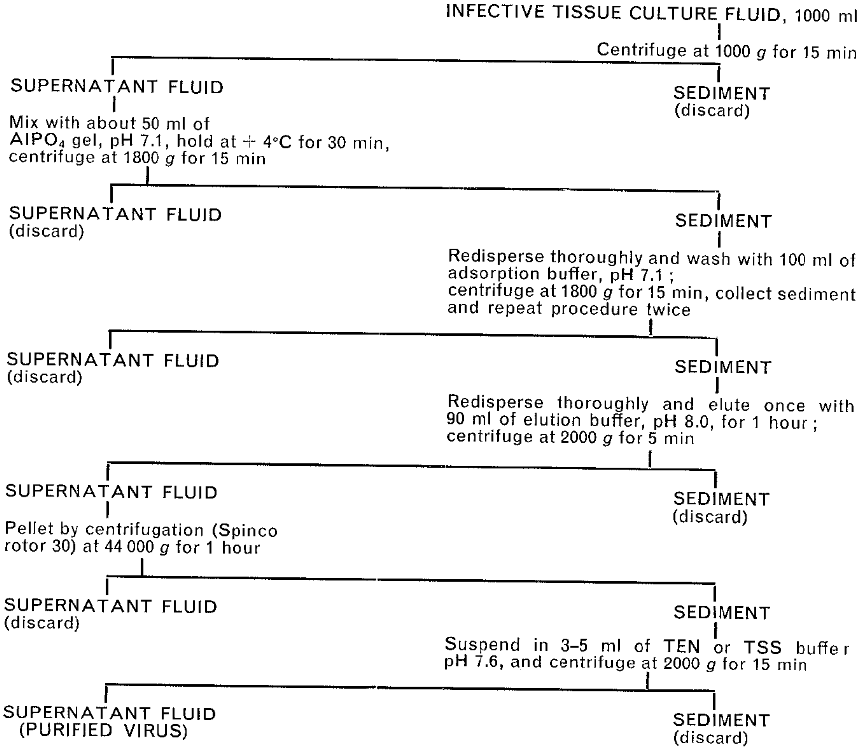
[1] Federal Research Institute for Animal Virus Diseases, Tübingen, Federal Republic

Aluminium phosphate gel

About 50 ml of a 16% (w/v) aqueous solution of trisodium phosphate dodecahydrate ($Na_3PO_4.12H_2O$) are added to 2 litres of distilled water and the mixture is treated with 50 ml of a freshly prepared 10% (w/v) solution of aluminium trichloride hexahydrate ($AlCl_3.6H_2O$), stirring slowly during the addition. (CAUTION : This operation should be performed under a safety hood.) The precipitate is sedimented by centrifugation for 5 min at 1800 *g* and is equilibrated by washing 3 times with adsorption buffer (pH 7.1–7.3). The quantity of gel thus obtained is sufficient for the treatment of 1 litre of tissue culture fluid.

For optimal adsorption aged aluminium phosphate gel is preferable to the freshly prepared material. "Aging" may be achieved artificially by autoclaving the freshly prepared gel. The aged gel may be stored for several months.

Concentration and purification procedure



INFECTIVE TISSUE CULTURE FLUID, 1000 ml

Centrifuge at 1000 *g* for 15 min

SUPERNATANT FLUID

SEDIMENT (discard)

Mix with about 50 ml of $AlPO_4$ gel, pH 7.1, hold at + 4°C for 30 min, centrifuge at 1800 *g* for 15 min

SUPERNATANT FLUID (discard)

SEDIMENT

Redisperse thoroughly and wash with 100 ml of adsorption buffer, pH 7.1 ; centrifuge at 1800 *g* for 15 min, collect sediment and repeat procedure twice

SUPERNATANT FLUID (discard)

SEDIMENT

Redisperse thoroughly and elute once with 90 ml of elution buffer, pH 8.0, for 1 hour ; centrifuge at 2000 *g* for 5 min

SUPERNATANT FLUID

SEDIMENT (discard)

Pellet by centrifugation (Spinco rotor 30) at 44 000 *g* for 1 hour

SUPERNATANT FLUID (discard)

SEDIMENT

Suspend in 3–5 ml of TEN or TSS buffer pH 7.6, and centrifuge at 2000 *g* for 15 min

SUPERNATANT FLUID (PURIFIED VIRUS)

SEDIMENT (discard)

More than 80 concentration-purification experiments have been performed to date in our laboratory, employing from 1 to 6 litres of infective tissue culture fluid per test. Recovery rates of 50–110% of virus infectivity (average 75%) were achieved after 200-fold to 300-fold concentration. Infectivity titres of virus purified by the aluminium phosphate method ranged from 1.3×10^9 to 1.3×10^{12} LD_{50}/ml.

The advantage over other concentration methods is the high degree of purity of the virus, which is free from bovine serum albumin. The protein content of the final preparation is reduced 2000-fold, on the average, in comparison with the starting material.

Average activity/protein ratios for the virus are shown in the table. From these data it was found that 1 LD_{50} corresponded to 10^{-14} g of protein, 1 HAU $\simeq 2\times10^{-8}$ g of protein, and 1 CFU $\simeq 3\times10^{-7}$ g of protein.

ACTIVITY/PROTEIN RATIOS OF RABIES VIRUS PURIFIED BY THE ALUMINIUM PHOSPHATE METHOD

Virus preparation	A	B	C	D	E	F	G	Mean values
µg of protein per ml	165	308	621	506	616	301	610	450
LD_{50} [a] per mg of protein	10.8	11.3	11.2	12.5	10.4	11.5	9.4	11.0
HAU [a] per mg of protein	5.7	5.4	5.3	5.7	5.0	5.1	4.8	5.3
CFU [a] per mg of protein	4.3	4.4	4.4	4.6	4.3	4.6	4.2	4.4

[a] $\log_{10}$ of actual values.

Virus purified by the aluminium phosphate method may be used advantageously for the production of highly potent antisera, as purified antigen for serological tests, and as starting material for physicochemical analysis of the virion or viral subunits, as well as for the production of a highly potent and purified antirabies vaccine.

A RADIOIMMUNE ASSAY FOR RABIES BINDING ANTIBODY

T. J. WIKTOR[1]

The radioimmune assay procedure described in this chapter provides a new immunological tool for detecting and evaluating rabies-specific antibodies by their capacity for binding to ^{125}I-labelled rabies virus antigen (Wiktor et al., 1972). This method appears to be more sensitive for detection of rabies antibody than any other procedure at present in use, and it should find useful application in the detection of the early antibody appearing after vaccination with the newly developed, concentrated, and purified rabies vaccines of tissue culture origin—antibody that cannot be detected by the conventional methods of virus neutralization, complement fixation, or fluorescent staining techniques.

Fundamental Principles

Trace labelling of antigens with radioactive isotopes can be used to simplify and increase the accuracy of immunological procedures. Much smaller quantities of reactants can be detected by this method than by non-isotopic methods.

Antigen-protein prepared in a pure state is labelled *in vitro* by the addition of a foreign atom, usually chromium or iodine. A carefully labelled protein, although essentially altered, cannot be distinguished from native protein by either *in vitro* or *in vivo* tests.

The method described by Hunter & Greenwood (1962) and modified by McConahey & Dixon (1966), using chloramin-T as the oxidizing agent to liberate elemental iodine from iodide, is the most convenient to use with high levels of activity, since it ensures minimum handling of radioactive material and little exposure to iodine vapour. As iodine will be substituted into the tyrosine fragment of all proteins that may be present, the product to be labelled should be as pure as possible to ensure the highest specificity.

In the system under consideration, only labelled antigen is determined and the reaction with antibody may be investigated if the labelled antigen bound to antibody can be separated from the unreacted free antigen. This separation is achieved by means of a second immunological step. In this "double antibody" system, the antibody immunoglobulin[1] in the form of a complex with the labelled antigen is precipitated by anti-immunoglobulin prepared in a different species. This method is based upon the finding that the antigenic properties of immunoglobulin are retained when it forms a complex with an antigen. For example, an antibody to rabies virus developed in rabbits will still be precipitated, after combining with rabies virus, by an anti-rabbit-IgG serum prepared in sheep.

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[1] The Wistar Institute, Philadelphia, Pa., USA.

Methods and Procedures

Antigen

A tissue-culture-adapted strain of rabies virus propagated in monolayer cultures of BHK-21 cells is concentrated by zinc acetate precipitation or ultrafiltration methods, as described in chapter 15, pages 166 & 167. The concentrated fluid is purified by two cycles of high-speed centrifugation (90 minutes at 35 000 *g*) and the final sediment is resuspended in 0.05 M phosphate buffer, pH 7, using a volume representing a 100-fold concentration of the original infectious fluid. The protein content is determined and the virus preparation is diluted with 0.05 M phosphate buffer, pH 7, to a concentration of 1 mg of protein per ml.

Iodination procedure

5 ml of virus are pipetted into a small (30-ml) beaker containing a magnetic stirring rod and placed in a crystallizing dish with cracked ice. The crystallizing dish is placed on a magnetic stirrer. 2 mCi of radioactive iodine, ^{125}I, in carrier-free form without preservatives or reducing substances, are added and the mixture is stirred well. While the protein-iodine mixture is being stirred, 0.5 ml of a freshly prepared solution of chloramin-T (1 : 5000 in distilled water) is added and stirring is continued for 5 more minutes. 0.5 ml of a freshly prepared solution of sodium metabisulfite (1 : 5000 in distilled water) is added so that any remaining oxidizing agent is neutralized, and the reaction is stopped. The virus is transferred to a dialysing bag and dialysed in the cold against 0.1 M phosphate buffer, pH 7, over a period of 12–18 hours with several changes of buffer. The total activity and the activity precipitable by trichloroacetic acid are determined. A good preparation will have a specific activity of $0.5–1.0 \times 10^8$ cpm per mg of protein, of which 95% will be precipitable by the trichloroacetic acid.

Anti-species-IgG-sera are prepared in rabbits or sheep by a series of inoculations of corresponding immunoglobulin emulsified in incomplete Freund's adjuvant.

Diluent

Dulbecco phosphate-buffered saline (PBS), pH 7.3, containing 0.1% of bovine serum albumin is used as the diluent throughout the test.

Radioimmune assay procedure

Serial fivefold or tenfold dilutions of inactivated normal and immune serum are prepared and 0.2 ml of each dilution is placed in one of a series of conical glass or plastic 15-ml centrifuge tubes. To each tube 0.2 ml of ^{125}I-labelled virus diluted to contain 5–10×10^4 cpm per sample is added and mixed. The mixture is incubated in a water bath at 37°C for 60 minutes and 0.1 ml of the corresponding anti-IgG-serum dilution is added to each tube (see Table 1) and mixed. The mixture is incubated at 37°C for 30 minutes and 0.1 ml of a 1 : 20 dilution of normal serum as a carrier protein is then added and mixed (see Table 2).

TABLE 1. DETERMINATION OF DILUTION OF ANTI-IgG-SERUM TO BE USED IN THE TEST

Anti-rabbit-IgG-serum dilution	Radioactivity (cpm per sample)						
	Normal rabbit serum			Immune rabbit serum			Difference (%)
	Total	Sediment	%	Total	Sediment	%	
1 : 32	108 684	10 806	9.9	104 415	17 808	17.0	7.1
1 : 16	105 828	10 387	9.8	102 747	14 869	14.4	4.6
1 : 8	96 353	16 191	16.8	102 424	22 738	22.2	5.4
1 : 4	100 873	34 207	33.9	104 717	82 911	79.2	46.3
1 : 2	103 743	33 115	31.9	105 003	85 242	81.2	49.3
1 : 1	102 088	34 370	33.7	101 317	73 430	72.5	38.8
1 : 1 (0.2 ml)	106 233	24 717	23.2	103 966	58 470	56.2	33.0

The maximum specific precipitation is given by the anti-IgG-serum at a dilution of 1 : 2, which will therefore be used in the test.

The mixture is incubated at 37°C for 30 minutes. A visible precipitate is formed immediately after addition of the carrier serum.

TABLE 2. DETERMINATION OF RABIES VIRUS ANTIGEN BINDING ACTIVITY IN ANTIRABIES RABBIT HYPERIMMUNE SERUM

Serum dilution ($\log_{10}$)[a]	Radioactivity (cpm per sample)						
	Normal serum			Immune serum			Specific binding %
	Total	Sediment	%	Total	Sediment	%	
0.7	80 385	26 349	32.7	81 754	64 013	78.3	45.6
1.4	81 674	25 952	31.7	80 968	58 702	72.5	40.8
2.1	79 987	24 383	30.4	80 237	57 449	71.6	41.2
2.8	79 873	24 284	30.4	79 952	55 246	69.1	38.7
3.5	80 527	25 263	31.3	80 243	45 337	56.5	25.2
4.2	81 091	24 642	30.4	81 294	30 566	37.6	7.2
4.9	80 583	24 964	31.0	80 782	26 496	32.8	1.8
5.6	79 963	23 940	30.0	81 221	24 853	30.6	0

[a] i. e., a dilution given as 0.7 = 1 : $10^{0.7}$.

The virus neutralizing activity of this serum was 1 : 2000 as measured by the 50% plaque reduction method.

The radioactivity of each sample is determined in a gamma counter.

Each tube is centrifuged at 1000 *g* for 30 minutes. The supernatant fluid is removed and the sediment is resuspended in 5 ml of diluent and centrifuged at 1000 *g* for 30 minutes. The supernatant is removed and the radioactivity of the sediment determined.

Evaluation of results

The specific binding activity of a test serum is determined after subtracting the background activity, determined from samples of normal serum treated in the same manner. The percentage of sedimentable radioactivity is calculated for each sample. The results are plotted as specific binding activity for each serum dilution.

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PART IV

METHODS OF VACCINE PRODUCTION

CHAPTER 18

GENERAL CONSIDERATIONS IN VACCINE PRODUCTION

K. HABEL[1]

The principles involved in the production of satisfactory rabies vaccines are no different from those considered generally applicable to any virus vaccine. The obviously important considerations are safety, potency, ease of production, and stability even after prolonged storage. To date, no one type of rabies vaccine has proved superior in all these characteristics, and there is therefore a choice among several products, each of which has some special advantage.

In general, with increasing emphasis on procedures aimed at increasing the safety of the product there has been a tendency for potencies to drop. This is seen in the duck-embryo vaccine where the small amount of nervous tissue used greatly reduces the hazard of postvaccinal complications of the central nervous system, but the immunogenic potency is usually somewhat less than that of the average brain-tissue vaccine. Recently, purification methods have been developed whereby the virus can be separated from all tissue materials, and it is even possible to isolate the antigenic part of the virus particle from the infectious virion (see Chapter 15).

Preservation of potency on prolonged storage can be achieved by lyophilization. Formerly this was possible only with vaccines inactivated by a virucidal agent that could be removed (ultraviolet irradiation, β-propiolactone); this technique has now been extended to the phenolized vaccines also.

Live virus vaccines, using virus grown in the chicken embryo, have proved superior to inactivated vaccines in veterinary use. These vaccines contain rabies virus strains attenuated by avian embryo or tissue culture passage to the point where they do not produce clinical infection on inoculation of the proper species, yet are still capable of multiplying in the vaccinated host. However, both these properties vary in different species. Although attenuated for dogs, the LEP Flury vaccine cannot be used in cattle for here it is still virulent, while the HEP Flury vaccine is avirulent for this species. On the other hand, the chicken-embryo live virus vaccines have limited advantages in man, for here the attenuated vaccine virus fails to ability to immunize different animal species emphasizes the need to use well-established strains, such as the Flury strains, for routine live virus vaccine production. In the case of live virus vaccines, the great importance of obtaining the necessary infective virus titre in production lots, and of lyophilizing the vaccine to preserve this titre, is obvious. If at the time of field use there is not sufficient viable virus to initiate the infection necessary for immunization of the vaccinated animal, these vaccines will not protect.

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[1] *Formerly* Department of Experimental Pathology, Scripps Clinic and Research Founda-

Present evidence indicates that rabies viruses throughout the world and from a variety of species have the same general antigenic structure, so there is no particular reason to use a local strain in vaccine production. On the other hand, there is a great advantage in using established standard strains, because extensive experience of laboratory production and field use, which already exists with these strains, is difficult if not impossible to obtain with newly developed strains.

Recent experimental studies suggest that on repeated passage transfers in cell cultures of tissue not derived from the nervous system, most strains of rabies virus become attenuated for experimental animals. Many of these attenuated strains are capable of immunizing animals. Thus there are numerous possible sources of live virus vaccines for use in animals, but because of the lack of experience in their practical application it is wise to continue using the established standard strains. The fact that viruses in general, and rabies virus strains in particular, tend to vary in their characteristics on growth in different cells, brings out the importance of using the seed virus to produce any vaccine. In general, virus for vaccine production should come from a frozen working pool made from a frozen or lyophilized standard pool kept for long-term use. The virus should not be passaged many times or in different species between removals of portions to serve as inoculum for vaccine production. The maintenance and use of the virus at the particular passage level at which field experience has been accumulated is of special importance in seed for live virus vaccines.

In the case of vaccines prepared from virus grown in animals, the quality of the animals is of obvious importance. In general, animals should be from controlled stock, in good health, and free from known latent infections. For vaccine prepared from the brains of suckling animals, the age of the animal at the time the brain is harvested is the crucial factor determining whether the material responsible for allergic encephalitis reactions to vaccine is present in the central nervous system tissue. The temptation to use older animals to meet production requirements and to obtain larger volumes of vaccine must be carefully avoided; otherwise, accidents may be expected to occur.

In general, rabies virus is a relatively poor antigen in inactivated vaccines. This is why the presence in the final vaccine of a large amount of the antigen must be ensured by using a virus source of high titre for inactivated vaccine production. Materials having an infectivity titre of less than 10^5 LD_{50} per 0.03 ml (for the mouse inoculated intracerebrally) will not make an inactivated vaccine of satisfactory potency. This low antigenicity is the reason for large vaccine doses, repeated inoculations, and difficulty in retaining potency after attempts at purification. Yet in spite of this, the efficiency of booster doses of rabies vaccine has been definitely established and basic immunity may persist for many years. This quantitative aspect of making potent vaccines is pertinent to the present requirement for multiple doses in postexposure prophylaxis. With newer methods using relatively high concentrations of purified antigen, the increased unit cost may be compensated by a reduction in the number of doses required.

There are a number of types of inactivated virus vaccine that appear satisfactory. In general, any method is acceptable that gives a safe and quantitatively proven potent vaccine. However, the ideal vaccine would be prepared from virus source material containing no proteins foreign to man, would be easily and inexpensively produced in large volumes with uniformly high titres, and would be inactivated by a method that consistently retained high immunizing potencies ; furthermore, it would be capable of long-term storage without a decrease in potency, would cause no reactions on repeated inoculation of patients, and would be effective with fewer doses. Since none of the vaccines at present available can meet all these requirements, research for vaccine improvement continues.

SEMPLE-TYPE VACCINE

B. SELIGMANN, Jr[1]

The following procedure is recommended to meet the requirements of the US Public Health Service[2] and is based on successful results obtained by vaccine manufacturers in the USA. Although rabbits are used exclusively in the USA for the manufacture of brain-tissue vaccine for administration to man, sheep and goats may also be used.

Formula of Vaccine

Each individual human injection of finished killed virus vaccine should contain not less than 2.0 ml of a 5% suspension of inactivated rabies fixed virus in phosphate-buffered saline (pH 7.0), or its equivalent in another concentration. The finished vaccine should contain not more than 0.25% of phenol for vaccines containing 10% or less of brain tissue or not more than 0.4% of phenol for vaccines containing more than 10% of brain tissue, as calculated from the amount originally added. In addition, either 0.008% of phenylmercuric borate or 0.01% of thiomersal[3] should be added.

Preparation of Seed Virus

Virus strain

The production strain used in the USA is a strain of fixed rabies virus selected on the basis of the high antigenicity of vaccines produced from it when challenged with a variety of other strains. This strain has been designated the PV strain.[4]

Maintenance of virus strain

Virus passage of the seed strain used for vaccine production is always through rabbit brain. A 10% suspension of infected rabbit brain in sterile distilled water is maintained in either the frozen or freeze-dried state.

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[1] Director, Division of Control Activities, Bureau of Biologics, Food and Drug Administration, Rockville, Md., USA.

[2] United States National Institutes of Health (1953) *Minimum requirements : rabies vaccine*, 3rd rev., Bethesda, Md.

[3] International Nonproprietary Name ; also known as thimerosal and merthiolate.

[4] Available to national laboratories upon request from the World Health Organization, 1211 Geneva 27, Switzerland.

Intracerebral inoculation of 0.25 ml of a 10^{-2} dilution of this preparation should produce paralysis in rabbits within 5–7 days after injection. Seed virus may be made as a 10% suspension of infected brains in sterile distilled water distributed into ampoules, glass-sealed and quick-frozen. The ampoules are preferably stored in a dry-ice[1] chest (−70° C), but in any case at a temperature lower than −15°C. The storage period may be as long as full potency is maintained as shown by mouse titration. The seed virus suspension should be of such activity as to kill all mice inoculated intracerebrally with 0.03-ml quantities in a dilution of not less than 10^{-6}.

The specificity of the virus strain is tested periodically by titration in mice against specific antirabies hyperimmune serum that has proved protective against street virus (see chapter 40).

Production of seed-virus 10% suspension

Rabies-infected rabbit brains are harvested from rabbits that have been inoculated intracerebrally with rabies virus and that have shown typical symptoms of rabies. The brains are harvested after the animals have developed complete paralysis but just prior to death. The brains are quick-frozen immediately and stored in a dry-ice chest until needed. If processed without freezing, all steps should be done as rapidly as possible, keeping the temperature as low as practicable. The brains are weighed and emulsified in a small volume of cold, sterile distilled water. Emulsification is effected in a Waring Blendor of appropriate size.[2] The frozen brains should preferably be minced by the Blendor blades and then a portion of the cold distilled water added for emulsification. Blending should be done in such a way as to minimize the rise in temperature. A total time of 4–5 minutes is usually all that is necessary. When the brains have been emulsified to a smooth paste, the rest of the cold distilled water can be added to yield a final 10% suspension (w/v) and blended briefly. The suspension is placed in appropriate volumes in ampoules, flame-sealed, and quick-frozen in dry-ice and alcohol. The ampoules are preferably stored at dry-ice temperature (−70°C), but in any case at a temperature lower than −15°C.

Sterility

The seed lot should be free from bacterial and fungal contaminants as indicated by an appropriate test (see below).

Virus titration

At least 3 intracerebral mouse titrations of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions are performed. The test period is 14 days, and all mice receiving can be expected that all mice receiving the 10^{-6} dilution will also die.

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[1] Solid carbon dioxide

Inoculation and Harvest

Preparation of inoculum and inoculation technique

The 10% suspension of seed virus is considered to be the 10^{-1} dilution and from this a final 10^{-3} solution of virus is made in 2% normal horse serum in distilled water. It is not necessary to centrifuge this preparation. A fresh dilution is made for each day's inoculation.

Healthy rabbits are firmly secured or lightly etherized and 0.25 ml of the inoculum is introduced by a sterile 2-ml syringe using a 10 mm × 0.90 mm ($^{3}/_{8}$-in, 20-gauge) needle through the skull approximately halfway between the outer canthus of the eye and the ear (see figure). A stainless-steel awl or a punch may be used to make the puncture in large rabbits.

Progress of disease in rabbits

Rabbits show marked signs of paralysis by the 4th day or occasionally as late as the 7th day following inoculation. The infected animal should show symptoms of fixed rabies virus paralysis and be moribund before the brain is harvested.

Killing of rabbits

Moribund rabbits that are completely paralysed but still breathing are killed by intravenous injection of approximately 20 ml of air into the marginal vein of the ear. Death occurs in 1–2 minutes. The brains from freshly killed rabbits only are used for vaccine production ; animals already dead from rabies or other causes are not used.

Autopsy

To guard against the use of unsuitable rabbits, autopsies are performed after brain removal. The brains of rabbits showing the presence of coccidiosis elsewhere in the body are not used because of the chance that coccidiosis rather than rabies may have caused paralysis. A record is made of all autopsy findings.

Brain harvest

The rabbit is washed with 5% phenol or another suitable antiseptic solution. Decapitation is performed as close to the shoulders as possible ; the severed head is fastened securely into a suitable holder, swabbed with tincture of iodine, and taken into a "sterile room" where actual brain removal is performed.

The skin is slit down the middle of the head and folded back and the exposed top of the skull is washed with tincture of iodine. The top of the skull is then removed with sterile bone-shears, and the brain is transferred with sterile scissors and forceps into a suitable sterile container. A small piece of the brain tissue is removed and tested for bacterial sterility. Brains should be quick-frozen as soon as harvested and stored at −15°C or lower, preferably in a dry-ice chest, until needed for vaccine production. Any brain showing bacterial contamination is discarded.

Manufacture of Vaccine : 40% Suspension

Emulsification

Frozen brains are weighed and placed in a 2-litre conical flask as a 40% suspension in sterile phosphate-buffered saline solution containing sufficient phenol to give a final concentration of 0.5% (see under *Diluent* below). After the brains have thawed, the flask is shaken by hand until they are well broken up. This partial emulsion is then transferred to a sterile Eppenbach colloidal mill (set at 22–24). Emulsification is completed by one run through the mill. A Waring Blendor or other suitable emulsifier can be used ; care should be taken to avoid running the emulsifier long enough to generate appreciable heat. From 4–5 minutes are usually sufficient for a Waring Blendor to emulsify the brains adequately. The container can be surrounded with a jacket of chipped ice and salt to prevent overheating of the contents ; a tight lid should be used to prevent dispersion of aerosol.

Diluent

(*a*) Phosphate-buffered saline solution : prepare a solution containing 2 parts of 0.5 M disodium hydrogen phosphate and 1 part of 0.5 M potassium dihydrogen phosphate.

To 1 part of this solution add 4 parts of 0.85% sodium chloride solution of pH 7.0.

(*b*) To make a 40% tissue concentration containing 0.5% phenol, add 0.68% of 90% phenol (laboratory reagent grade) to phosphate-buffered saline solution (this allows for the volume of emulsified brain tissue) ; make a 40% (w/v) suspension with the diluent according to the weight of the brains.

NOTE : After the phenol has been added, great care should be taken not to freeze the brain-tissue suspension, either as the concentrated 40% form or as the final vaccine dilution. *Freezing of phenolized suspensions*

A sample of 40% tissue suspension is weighed (because of air content from action of the mill), diluted immediately, and titrated in mice weighing 11–15 g. Dilutions of 10^{-6}, 10^{-7}, and 10^{-8}, representing dilutions of brain-tissue content, are injected intracerebrally and the titre (LD_{50}) should be at least 10^{-6} (see Appendix 1, page 321 for calculation).

Incubation

The emulsified vaccine containing 0.5% of phenol is brought to a temperature within the range 20–30°C in a water-bath for 1 hour and then placed in an incubator within the range 20–30°C for 72–76 hours or until the virus has been inactivated as demonstrated by the innocuity test. The vaccine is agitated mechanically during the entire period in the incubator.

NOTE : The period in the incubator required for inactivation will vary with different virus strains and methods of emulsification of brain tissue (fineness of particles). The time required should be established in each laboratory through experimental trials, e.g., intracerebral inoculation of mice with suspension periodically during incubation.

Dilution to 20% vaccine

The 40% vaccine is diluted with an equal volume of the sterile phosphate-buffered saline containing 1 : 5000 thiomersal, so that the finished product contains 20% of tissue, 0.25% of phenol, and 1 : 10 000 thiomersal.

Diluted vaccine is passed through several layers of gauze or silk filter for the purpose of removing any unground particles of brain tissue. A convenient batch of vaccine is about 2 litres in volume. It is subjected to the safety and sterility tests described below, and is stored at 4–6°C.

Sterility test on bulk vaccine

A sterility test on the bulk vaccine is made in fluid thioglycolate medium. A representative sample of at least 10 ml from each container of bulk vaccine should be tested. The sample should be subdivided or placed in a sufficient volume of the test medium to dilute the phenol to a concentration not greater than 0.008% in each container of sterility test medium. Tests in fluid thioglycolate medium are incubated at 30–32°C for not less than 7 days and examined visually for evidence of growth periodically during this time. In order that growth shall not be overlooked because of turbidity, on the 3rd, 4th or 5th day and on the 7th day of incubation the test containers are mixed and 1 ml from each container is inoculated into additional containers of sterile test medium. All containers are incubated for an additional 7 days. Any vaccine showing contamination is retested, using a double volume for the test.

Innocuity test on bulk vaccine

An innocuity test is performed both in rabbits[1] and in mice to confirm the absence of viable virus in the bulk vaccine. This test should be performed on a representative sample taken from each container of bulk vaccine. Not less than 2 animals of each species are used for each test. Each rabbit is injected intracerebrally with not less than 0.25 ml and each mouse (weight 18–20 g) with 0.03 ml of vaccine containing not less than 5% of brain tissue. All test animals are observed for not less than 14 days during which time each animal must remain free from symptoms of rabies or of other central nervous system disease.

Because it is difficult to obtain infection of 100% of rabbits inoculated with rabies virus suspensions, even when using material with a relatively high titre, it is preferable to have the innocuity test performed by a person who has regular experience in the inoculation of animals with rabies virus and who consistently obtains high percentages of infection. This provides greater assurance that the test is performed in a manner that will ensure maximum sensitivity.

Finished vaccine

After all tests performed on the bulk vaccine have been completed satisfactorily, the vaccine is filled into final containers and labelled.

Representative samples are taken for the following tests:

Analytical tests

Appropriate tests are performed to determine the total solids, phenol concentration, pH, and thiomersal or other preservative concentration.

General safety test

A safety test for the detection of any inadvertently added toxic substances is made on the undiluted contents of final containers selected at random from each filling of each lot. The parenteral injection of 0.5 ml for mice weighing approximately 20 g each and 5.0 ml for guineapigs weighing approximately 350 g each should cause neither death nor significant signs. At least 2 animals of each species are used, and the observation period is not less than 7 days.

For the sterility test and first repeat sterility test on the finished vaccine, samples should be taken from not less than 20 final containers from each filling of each lot, selected to represent all stages of filling from the bulk container. If the amount of material in the final container is 1.0 ml or less, the entire contents should be tested. If the amount of material in the final container is more than 1.0 ml, the volume tested should be the largest single dose recommended or 1.0 ml, whichever is larger. In addition to fluid thioglycolate medium used as described under *Sterility test on bulk vaccine* (above), a test should be performed in fluid Sabouraud's medium for the detection of mould contaminants. Incubation for this medium should be 20–25°C with an observation period of not less than 10 days; a period of up to 21 days is necessary for the development of some contaminants. Experience has indicated that the volume of inoculum and medium should be adjusted so that mercurial preservatives will be diluted to less than 1 part in 7 million in order to prevent inhibition of growth in this medium. As an alternative, fluid thioglycolate may be used and incubated at 20–25°C.

Potency

See chapter 33, page 283.

Dating

The expiry date is not more than 6 months after the date of manufacture or date of issue. The date of manufacture is the date of the initiation of the last satisfactorily passed potency test. The date of issue may be 3 months after the date of manufacture, provided that the vaccine has been stored at 2–5°C by the manufacturer for this period. The product should be stored at 2–5°C at all times. Freezing of phenolized rabies vaccines destroys the potency.

CHAPTER 20

FERMI-TYPE VACCINE

P. LÉPINE[1]

An advantage of the Fermi-type vaccine is ease of preparation : it is possible to produce very rapidly a vaccine whose potency can be grossly assessed from the titre of residual virulence when there is no time to carry out a full potency test. However, several laboratories have recently changed over to the manufacture of completely inactivated vaccines. The Pasteur Institute in Paris, for example, has completely discontinued the use of Fermi-type vaccine since January 1967 and now produces a freeze-dried β-propiolactone-inactivated vaccine that ensures much higher potency, can be kept for a longer period, and is more resistant to the high temperatures encountered in tropical countries (see chapter 21 (B), page 204).[2] Consequently, only a brief description of the Fermi-type vaccine is provided in the present edition of this monograph. A fuller account may be found in the second edition.

Formula of the Vaccine

The vaccine consists of a 5% aqueous suspension of brain tissue from sheep or goats that have been inoculated with fixed rabies virus. The suspension is treated with phenol at an initial concentration of 1%, but this may be reduced to 0.5% in the final vaccine. Although Fermi-type vaccine is usually classed as an inactivated vaccine, it contains a precisely determined quantity of active virus in addition to the inactivated virus. It is thus a mixed attenuated-inactivated type of vaccine.

Production

For the rabies strain employed, the preparation of seed virus, the inoculation of sheep, the removal of the brain, and the sterility and infectivity tests, see chapter 21(B) (pages 204–210).

Inactivation

The vaccine mixture containing a homogeneous suspension of 10% of brain tissue and 1% of phenol (diluent : buffered saline, pH 7.4–7.6) never be carried out at a temperature below 22°C. A vaccine that has been inactivated for 24 hours at 22°C and then kept for 4 days at 4°C may subsequently be diluted to give a brain-tissue concentration of 5% and a phenol concentration of 0.5%. It is then dispensed into 5-ml ampoules. The bulk vaccine is stirred constantly during the filling process.

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[1] Pasteur Institute, Paris, France.

To complete the inactivation of the virus, the filled ampoules are kept for at least a week at 2–5°C before being sent to the packing department.

Tests on the Final Vaccine

Each final lot of vaccine must be tested in mice to determine its residual virulence. This test should be performed 9 days after the addition of phenol. In cases of exterme urgency it may be performed sooner, but not less than 2 days after the addition of phenol.

A sample of the vaccine to be tested is submitted to low-speed centrifugation and the supernatant diluted 10^{-1}, 10^{-2}, and 10^{-3} with physiological saline, pH 7. Each dilution is administered by intracerebral inoculation to a group of at least 5 (preferably 10) mice, each animal receiving 0.03 ml. The 3 groups of mice are kept under observation for 15 days and a record is made of the number of paralysed animals that die from the 3rd day onwards.

The residual virulence of the vaccine should be such that

(*a*) all animals die with the 10^{-1} dilution ;

(*b*) the mortality with the 10^{-2} dilution is more or less constant (not more than 50%) ;

(*c*) no deaths occur with the 10^{-3} dilution ;

(*d*) the virus titre, as calculated by the methods given in Appendix 1, page 321, is not greater than $10^{-2.7}$.

Tests for sterility and potency and analytical tests (pH, phenol content) should be carried out on the same lines as the tests described in chapters 21(B) and 31.

Expiry Date

The expiry date is 5 months from the date of issue, provided that the vaccine is kept at a temperature between 2°C and 5°C throughout this time. If low-temperature storage cannot be guaranteed, the expiry date is reduced to 2–3 months from the date of issue, depending on the climate.

CHAPTER 21

PHENOLIZED, FREEZE-DRIED SHEEP BRAIN VACCINE

A. METHOD USED IN THE USSR

M. A. SELIMOV [1] *& V. M. MOROGOVA* [2]

The vaccine described in this chapter has been in use in the USSR since 1966 (Selimov et al., 1961).

Composition

The vaccine is a freeze-dried product containing 5% of a sheep brain suspension of fixed rabies virus, 3.75% of sucrose, and not more than 0.25% of phenol.

Preparation of the Seed Virus

Fixed rabies virus originating from the Pasteur strain is used for the production of rabies vaccine in the USSR. Passages 3249 and 3251 of this "Moscow strain" are sent annually in dried form to all manufacturing institutes by the L. A. Tarasevič State Control Institute.

The strain is passaged in rabbit brain not more than once every 2–3 years in the State Control Institute and not more than once a year in production. Portions of rabbit brain infected with the fixed rabies virus are stored in chemically pure, neutral 50% glycerol at 4°C or in undiluted glycerol at —40°C. Before the sheep are infected, the rabbit brain tissues are carefully washed in meat-peptone broth or distilled water (pH 7.2) to remove the glycerol. The tissues are then ground to a homogeneous mass and a 10^{-2} dilution is prepared ; it should have a titre of 6.0–6.2 log LD_{50} in 0.03 ml. The seed virus is tested for sterility.

Preparation and Infection of the Sheep

Healthy sheep between 4 and 12 months old are used in vaccine production. The animals are obtained from farms known to be free from infectious diseases and are kept in quarantine for 30 days.

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[1] Professor and Head, Rabies Prophylaxis Laboratory, Institute of Poliomyelitis and Viral Encephalitic Diseases, USSR Academy of Medical Sciences, Moscow, USSR.

oughly disinfected and an electric trephine is used to make a hole in the skull without previous incision. A sterile syringe is used to inject 0.4–0.5 ml of the suspension containing the virus into the brain. Not more than 12 sheep are infected at one time.

The sheep are killed by severing the cervical blood vessels when clinical evidence of laboratory rabies appears 80–144 hours after infection. After the head has been skinned and the muscles removed it is treated with a 5% solution of phenol and inserted through a special aperture into a box. It is then flamed with phenolized alcohol and separated from the carcass. After the cerebrospinal canal has been plugged with sterile cotton wool, the head is transferred to a sterile gauze cloth, placed in a sterile container, and sent to the production department. A pathological examination is performed on the carcass.

Removal of the Brain

Normally the vaccine is prepared immediately after the animals have been slaughtered, but under exceptional circumstances the head or brain is stored at 2–4°C for a maximum of 12 hours. The head is firmly secured, wiped, and flamed with alcohol, after which the skull is opened with an electric saw. Samples of brain are taken for cultivation to test for sterility, and the brain is cut into pieces, removed, placed in a sterile jar, and weighed.

Preparation of Vaccine

An electric blender or shaker is assembled, sterilized in an autoclave, and connected to a graduated reservoir containing a 1% solution of double-distilled phenol in physiological saline. The blender is also connected to a bottle for receiving the vaccine. It is then closed and 100 ml of the phenolized saline are run in from the reservoir, after which 1–3 rabbit brains are introduced through the loading aperture. Grinding takes 10–12 minutes, during which time no heating of the blender or the vaccine should occur.[1] Sufficient 1% phenolized saline to form a 20% brain suspension is added in two portions and the vaccine mixed by switching on the blender. It is then filtered under suction through a double-walled muslin bag into the receiver. When filtration is complete, the bottle is disconnected and a sample siphoned off for sterility testing.

The 20% vaccine with 1% of phenol is inactivated by placing in an incubator at 22°C for 14 days, shaking two or three times a day. It is then suspended in an equal volume of 15% sucrose in double-distilled water. This gives a 10% vaccine with 0.5% of phenol and 7.5% of sucrose, which is shaken continuously and dispensed in 1.5-ml amounts into 6-ml vacuum ampoules. Each ampoule is loosely plugged with cotton wool. When the entire batch has been filled into ampoules, the vaccine is shaken well and cooled to between −40°C and −50°C. After being kept at this temperature for 18–20 hours, it is transferred rapidly to the vacuum drying apparatus. Freeze drying is performed at a pressure of 0.007–0.002 mm Hg and takes 40–48 hours. After 18 hours the temperature is allowed to rise gradually for 2 hours until it reaches 22–24°C and is then kept at this level for the rest of the freeze-drying operation. The ampoules are subsequently sealed in the vacuum dryer at room temperature under a pressure of 0.01 mm Hg. Each ampoule is supplied with 3 ml of pyrogen-free distilled water for reconstitution of the vaccine.

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[1] It is recommended that an ice-jacket be used to prevent overheating and that the container be fitted with a tight lid to prevent dispersion of aerosol (cf. chapter 19, p. 195).

Tests on the Final Vaccine

(1) Determination of physical properties (appearance and solubility). The vaccine should dissolve in 1–2 minutes.

(2) Sterility test on the vaccine (10 ampoules from each batch).

(3) Sterility test on the reconstitution fluid.

(4) Bacterioscopic examination of (*a*) the reconstituted vaccine after shaking, (*b*) the supernatant liquid after centrifugation for 40 minutes at 2500–3500 rev/min, and (*c*) the sediment after centrifugation.

(5) Safety test on 5 mice, by subcutaneous injection of 0.5 ml of 5% vaccine from each batch. The mice should survive for 5 days and no areas of infiltration should develop.

(6) Test for residual virulence. 1.5 ml of distilled water are added to each of 3 ampoules containing dry vaccine. The 10% vaccine thus obtained is centrifuged at 1000 rev/min for 15 minutes. The supernatant liquid, which is a 10^{-1} dilution, is used to prepare dilutions of 10^{-2} and 10^{-3}. Each dilution is administered intracerebrally (0.03 ml) to 6 mice weighing 10–12 g. Observation is continued for 14 days. Mice that die before the 5th day are not taken into consideration in the test. The residual infective titre, as calculated by the Reed & Muench or Spearman-Kärber method (see Appendix 1, page 321), should not exceed 10^{-2} LD_{50} per 0.03 ml;

(7) Potency test. The immunogenicity of each batch is tested by Habel's method or by the USPHS technique using the reference vaccine of the L. A. Tarasevič State Control Institute. The indices of the dry vaccine should be not less than 1000 if Habel's method is used and not below the limit set for the batch of reference vaccine employed if the USPHS method is used (see chapter 33).

(8) Determination of residual moisture content. This is done by measuring the difference in the weight of a sample before and after one hour in a desiccator at 100°C. The difference should not exceed 3%.

The expiry date of the freeze-dried vaccine is 2 years from the time of the potency test.

REFERENCE

Selimov, M. A., Boltutsi, L. G., Semenova, E. V. & Ponjatovskaja, L. D. (1961) *Ž. Mikrobiol. (Mosk.)*, 5, 46

B. METHOD USED AT THE PASTEUR INSTITUTE, PARIS

P. LÉPINE,[1] *P. ATANASIU,*[2] *A. GAMET*[3] *& C. VIALAT*[4]

Principle

The vaccine is prepared from the brains of very young sheep inoculated with a strain of fixed virus. The pulped nervous tissue is suspended in a buffered isotonic diluent so that it can be lyophilized without loss of antigenicity. The fixed virus is inactivated by the addition of β-propiolactone. All the procedures must be carried out with strict aseptic precautions in a sterile atmosphere, which can be achieved by use of a closed system.[5]

Fixed Virus Strain Employed

The strain of fixed virus used for inoculating the young sheep must be of high antigenicity and all its characteristics must be subject to periodic control. Either the original Pasteur strain or one of its daughter strains may be used. In the Pasteur Institute, Paris, the lyophilized VP11 (Pasteur Virus No. 11) strain is used. Every year a number of adult rabbits weighing 2 kg are inoculated intracerebrally with this strain. When the infection is fully developed, the brains are harvested while the animals are lying paralysed. After the brains have been ground up, a 10^{-1} suspension is prepared with the usual diluent (distilled water containing 2% of horse serum), dispensed into ampoules, and stored at $-20°C$. This constitutes the stock of inoculum required, thus avoiding the need for multiple passages in the rabbit.

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[1] Pasteur Institute, Paris, France.

[2] Head, Rabies and Rhabdovirus Research Laboratory, Pasteur Institute, Paris, France.

[3] Head, Antirabies Centre, Pasteur Institute, Paris, France.

[4] Pasteur Institute, Paris, France.

[5] In the concentration used β-propiolactone is only slightly bactericidal, so that if the manufacturing conditions are not sufficiently sterile it will be necessary to add an antimicrobial substance. Phenol is recommended because of the long experience of its use in the production of rabies vaccines.

Recovery of Rabid Sheep Brains

Only very young sheep must be employed, aged 1–2 months at most. During the weeks following the lambing season, a considerable stock of rabid brains should be accumulated so that vaccine production can continue throughout the year : brains stored dry at −25°C can be kept for a year without change in virus titre.

The young sheep are kept in quarantine under veterinary supervision for one week, during which time they are observed and prepared for inoculation. Two days before inoculation, a depilatory paste is applied between the orbital arch of the frontal bone and the base of the ear. This provides a bare surface that can readily be disinfected ; tincture of iodine is applied 3 times before inoculation.

The inoculum is prepared just before use by thawing out an ampoule of the deep-frozen 10^{-1} virus suspension. The sheep is firmly secured on a special table and the inoculation area is once more disinfected with tincture of iodine. A hole is made in the left lateral part of the frontal bone with a Besredka trephine, so that 0.5 ml of the inoculum can be injected into the brain.

After inoculation, the sheep are placed in isolation pens (not the same pens as those used for quarantine). On the 5th day after inoculation they show the first signs of disturbed equilibrium. On the 6th day, when they are paralysed, they are killed by total exsanguination and decapitated by section of the posterior part of the cervical column. The heads are skinned, placed on a sterile tray, sprayed with tincture of iodine, and flamed with a bunsen burner. In a sterile room, with all the usual precautions to ensure aseptic conditions and to protect the operator and his assistants (gloves, goggles), a wide lozenge-shaped opening is made in the calvaria. After incision of the meninges, the whole of the brain is removed and placed in a sterile jar of a type that can be hermetically sealed.

A sample is taken for sterility testing and for determination of the virus titre, after which the jar is stored at −25°C. The sterility tests are carried out in Bonnel & Raby media for aerobic and anaerobic organisms. Virus titration is carried out by intracerebral inoculation of white mice weighing 14–16 g : the titre should be about $10^{-5.5}$ LD_{50} per 0.03 ml of inoculum. Brains found to be sterile and with an adequate virus titre may be kept for up to a year at −25°C.

Preparation of Vaccine

The buffer solution from which the suspending medium for the vaccine is prepared has the following composition :

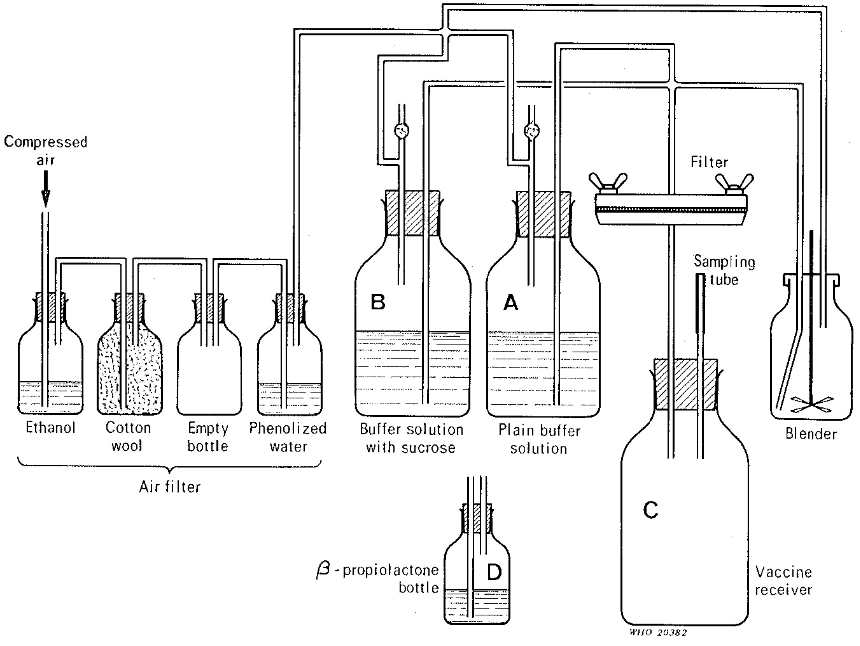
sodium chloride	5.85 g
sodium dihydrogen phosphate dihydrate	6.24 g
sodium hydroxide pellets	1.62 g

lization is prepared by adding sucrose to this buffer solution in the proportion 75 g of sucrose to 1000 ml of buffer.

The apparatus used for preparation of the vaccine is shown in the accompanying figure. Half the total quantity of buffer solution needed is placed in bottle A and the other half in bottle B, together with the appropriate quantity of sucrose for the finished vaccine. Thus, for the preparation of 8 litres of vaccine, two 10-litre bottle are used, one containing 4 litres of plain buffer solution and the other 4 litres of buffer solution plus 600 g of sucrose. The two bottles and the blender used for homogenizing the brains are connected by a system of tubes in the manner shown, so that the whole manufacturing operation can take place in an atmosphere of sterile compressed air, thereby excluding the possibility of bacterial contamination. The complete assembly, including the buffer solutions, is sterilized by autoclaving at 105°C for 60 minutes.

The sheep brains are removed from the refrigerator, thawed rapidly, and weighed out in portions of 400 g, corresponding to the capacity of the rotary blender. A small quantity of the plain buffer solution from bottle A is admitted to the blender, which is then allowed to run at a maximum speed of 10 000 rev/min for 2 minutes. More of the same solution is then added and the blender is run for a further 30 seconds, but at its minimum speed, in order to homogenize the mixture. This is then transferred to

APPARATUS FOR THE PREPARATION OF PHENOLIZED SHEEP BRAIN VACCINE



bottle B, containing the buffered sucrose solution, by admitting sterile compressed air. The empty blender is rinsed by admitting more of the plain buffer, which is subsequently also transferred to bottle B. The process is then repeated with a second blender, again containing 400 g of rabid brain tissue, and the process is repeated using the two blenders alternately until the 4 litres of solution in bottle A have been used up and the 2 portions of brain tissue homogenate have been transferred to bottle B by the admission of sterile compressed air.

The 8 litres of vaccine emulsion thus obtained are then passed, by the same means, through a nylon filter with a calibrated mesh of 4.2 × 10 mm and collected in the sterile 10-litre bottle C. This contains a magnetized bar to permit the subsequent use of a magnetic stirrer, and is fitted with a two-holed rubber bung carrying a sampling tube and the tubing for the addition of β-propiolactone.

In addition, a small bottle (D) containing 250 ml of distilled water is fitted with a rubber bung provided with 2 tubes : one is for the sterile compressed air supply, while the other is used first for the addition of β-propiolactone and is then connected with the intake tube of the receiver C. This apparatus is sterilized at 105°C for half an hour at the same time as the apparatus described above. It is kept at 4°C while the vaccine emulsion is prepared. When this is ready, sufficient β-propiolactone (2 ml) is added to the sterile distilled water in the small bottle D to give a concentration of 1 : 4000 in the final vaccine emulsion. The solution is stirred rapidly for a few seconds and then the bottle is connected both to the compressed air supply and to the receiver C, which has meanwhile been placed on a magnetic stirrer. The entire β-propiolactone solution is then added to the vaccine emulsion and inactivation is allowed to proceed for 3 hours at room temperature, i.e., 22–24°C.

At the end of that time 100 ml of vaccine emulsion will contain :

rabid sheep brain		10 g
sodium dihydrogen phosphate dihydrate		0.624 g
sodium chloride		0.585 g
sodium hydroxide		0.162 g
sucrose		7.5 g
distilled water	to make	100 ml

The β-propiolactone disappears, being rapidly hydrolysed to wholly non-toxic derivatives of β-propionic acid and hydracrylic acid. During this process, it inactivates the fixed rabies virus. In the concentration of 1 : 4000, however, the bactericidal action of β-propiolactone is weak, and contamination may occur if the working conditions are not entirely adequate. This can be remedied by the addition at this stage of phenol to produce a 0.25% concentration (20 g of phenol for the 8 litres of vaccine).

The receiver C is kept at 4°C for 48 hours, which ensures total hydrolysis of the β-propiolactone and, if phenol has been added, allows sufficient

As soon as the vaccine emulsion is removed from the refrigerator, it is dispensed, under sterile conditions, in 2.5-ml quantities into 10-ml bottles. The bottles are then cooled abruptly to −40°C or below, so as to produce small crystals and thus ensure that the dried product will be homogeneous.

The drying process, which is conducted at a pressure of 0.2 mm Hg, takes 40 hours, starting with the vaccine at a temperature of not more than −40°C and allowing the temperature to rise slowly to + 22°C, at which level it is maintained throughout the last 10 hours. When lyophilization is completed the vacuum is destroyed by the admission of oxygen-free anhydrous nitrogen, and the bottles are sealed with a rubber stopper and a metal cap.

Reconstitution and Dosage

The thoroughly lyophilized vaccine has a porous, biscuit-like appearance and comes away from the sides of the bottle in large pieces. It can be reconstituted rapidly by adding distilled water and shaking gently for a short time ; the product obtained is perfectly homogeneous. The vaccine must be reconstituted immediately prior to use, by injecting 5 ml of distilled water through the stopper. This gives 5 ml of vaccine suspension containing 5% of nervous tissue.

The vaccine is administered by subcutaneous injection in daily doses of 2.5 ml for adults and 1 ml for children aged 0–5 years. The injections should be given in accordance with the treatment schedules recommended by the WHO Expert Committee on Rabies.[1]

This vaccine has no residual virulence and may therefore be used for intradermal booster injections. For this purpose a dose of 0.25 ml is equally distributed between 2 sites on the anterior surface of the forearm. This procedure is also recommended for preventive vaccination before exposure.

Expiry Date

When correctly lyophilized and nitrogen-sealed, the vaccine is perfectly stable at a temperature of 22–24°C for 2 years. Nevertheless, to counteract the effects of improper storage conditions, particularly in the tropics, *storage under refrigeration is recommended.* Kept under these conditions, the vaccine is usable for 2 years after the last satisfactory potency test.

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[1] See, for example, *Wld Hlth Org. techn. Rep. Ser.*, 1973, No. 523, Annex 1.

Tests on the Final Vaccine

With the exception of the fixed virus inactivation test, all tests are made on the final lyophilized product, reconstituted with sterile double-distilled water.

Determination of pH

This is done with indicator paper. The pH should be between 6.6 and 7.0.

Sterility test

This test is made on 10 bottles of vaccine reconstituted by the addition of 5 ml of sterile distilled water per bottle.

Detection of aerobic and anaerobic organisms

Three ml from each bottle are seeded into 2 tubes of Bonnel & Raby sodium hydrogen sulfite medium; one tube is incubated at 37°C while the other is kept at laboratory temperature (22°C).

The tubes are kept under observation for a week, after which the contents are cultured on agar-agar medium.

Detection of yeasts and fungi

The remaining 2 ml from each bottle are seeded on Segrétain malt extract medium and kept under observation for 2 weeks at laboratory temperature.

Virus inactivation test

This test is carried out on the vaccine emulsion after inactivation for 3 hours with β-propiolactone but before the addition of any phenol. This period should suffice for the elimination of any residual virulence. The sample is taken from the collecting bottle through the special tube provided, since opening the bottle might cause contamination.

The vaccine is inoculated intracerebrally into a group of 20 mice weighing 14–16 g, each animal receiving 0.03 ml. The mice are observed for 14 days; any deaths occurring during the first 4 days can be disregarded, but all mice must survive after this date. If there is any abnormal mortality, the test must be repeated on 40 mice, with 20 bottles of lyophilized vaccine.

Two complete (unmodified) Habel tests (see Chapter 31) are conducted in parallel, using only one group of control mice. In the first test, the mice are inoculated with lyophilized vaccine that has been stored at 4°C and in the other test they are immunized with vaccine from the same batch that has been incubated for 1 week at 37°C. In both tests the vaccine should be found to protect against at least 10^3 LD_{50}, and the potency in the first test should not be more than ten times that in the second test.

Test for encephalitogenic activity

The technique used is that described by Svet-Moldavskij et al. (1965).[1] Ten bottles of lyophilized vaccine are reconstituted ; the 50 ml of vaccine thus obtained are combined and centrifuged, and the precipitate is mixed with Freund's adjuvant. The preparation is then administered subcutaneously to 10 guineapigs weighing 400–500 g each in doses of 2.2 ml at each of 5 points on the abdomen. The guineapigs are placed under observation for 45 days and must show no signs of paralysis. Any animal that dies during the observation period is autopsied and the brain and medulla are examined histologically.

Detection of contaminants

Two very young sheep are each inoculated intracerebrally with 0.50 ml of reconstituted vaccine. The animals are kept under observation for 3 weeks and must remain healthy, otherwise this test is repeated on 4 animals.

Titration of β-propiolactone

The β-propiolactone used must be titrated on receipt and at yearly intervals thereafter to make sure that it contains at least 80% of active β-propiolactone. As already mentioned, β-propiolactone is very rapidly hydrolysed in a saline medium and is entirely absent from the finished vaccine. The titration is therefore carried out on the container of β-propiolactone itself, using the technique of Tyler & Beesing (1952), as follows :

Reagents

0.4 M	sodium thiosulfate
2 M	dipotassium hydrogen phosphate (K_2HPO_4) solution
1 M	sodium acetate solution
0.5%	starch solution
0.1 M	iodine solution

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[1] See also chapter 31.

Technique

With a pipette, measure out 25 ml of 0.4 M sodium thiosulfate and add 2 ml of the dipotassium hydrogen phosphate solution. Add not more than 0.5 g of the sample to be tested, containing about 5 mmol (0.3–0.4 g) of β-propiolactone. Shake gently to dissolve and wait 10–12 minutes. When the reaction is complete, wash the walls of the vessel with about 25 ml of water and titrate by means of the 0.1 M iodine solution, using the starch solution as indicator.

Control titrations

Blank titrations must be carried out at the same time and under the same conditions to correct the small errors that may occur in the titration of thiosulfate in a slightly alkaline solution. The β-propiolactone content (%) is given by the formula :

$$\frac{(A-B)\ N \times 7.206}{W \times 0.995}$$

where

A = ml of iodine solution required for the sample
B = ml of iodine solution required for the blank
N = normality of the iodine
W = weight of the sample
0.995 = correction factor

Free phenol content

If phenol is added during the preparation of the vaccine to remove contamination, most of it will be eliminated during lyophilization, but it is nevertheless necessary to determine how much remains. As a rule only traces will be found (0.1 g per 100 g at most).

Reagents

Folin-Ciocalteu solution (King & Wootton, 1959)
20% sodium carbonate decahydrate
stock reference solution of phenol, 1 : 1000
reference solution of phenol, 10 g/ml

Technique

Centrifuge the reconstituted vaccine at 10 000 *g* for 10 minutes, decant off the supernatant liquid and dilute it 1 : 1000 with demineralized water. Take 1 ml of this solution and make up to 10 ml with demineralized water. Add 0.5 ml of Folin-Ciocalteu reagent, mix, add 2 ml of the 20% sodium carbonate solution, and mix again. Leave in contact for 20–30 seconds, then place in a boiling water-bath for 1 minute. Cool. Make spectrophotometer readings at 310 nm against one blank solution and one control solution

The Pasteur Institute has recently begun the production of a vaccine similar to the above but using suckling mouse brain in place of sheep brain. The animals, which must be free from specific pathogenic organisms, are inoculated not more than 4 days after birth with a strain of fixed rabies virus and the brains are removed 4 days later. The vaccine is prepared as a 2% suspension, inactivated with β-propiolactone, with the addition of a protective, stabilizing diluent to permit lyophilization.

The daily dose is 2 ml in adults and 1 ml in children, a total of 7 subcutaneous injections being given. Booster injections are given either intradermally, in doses of 0.25 ml, or subcutaneously, in doses of 2 ml.

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CHAPTER 22

SUCKLING RAT BRAIN VACCINE

M. K. KARAKUJUMČAN,[1] B. M. PARIZ[2] & V. D. SOLOV'EV[3]

An important requirement for vaccine preparations is that they should maintain their specific activity over a lengthy period, not only at optimum temperature but also under less favourable environmental conditions. This is particularly true of antirabies vaccine, since it may have to be transported at any time to any place where it is urgently needed.

Liquid antirabies vaccines remain fit for use for a comparatively short period only. They are issued with a statement concerning the expiry date (see chapter 19, page 198) and the storage temperature, which must not exceed 5°C. This makes their storage, transport, and use appreciably more difficult. Freezing the vaccine or heating it above 37°C causes speedy coagulation of the tissue substrates and renders the vaccine unfit for use.

The method of freeze-drying has long been in use in the production of bacterial and viral vaccines, but the first freeze-dried antirabies vaccine for human treatment was developed in 1964 in the Moscow Institute for Research on Virus Preparations; subsequently this vaccine has been produced on a large scale. Studies on this vaccine undertaken in the USSR indicate that quite satisfactory results can be obtained from lyophilization of antirabies vaccine made from brain tissue if sucrose is added as a stabilizer.

Apparently, practical application of dried antirabies vaccine has been hindered by fears that it might cause more severe complications than those that sometimes arise following the use of liquid vaccines. The authors have therefore devised a method of producing dried antirabies vaccine by using the "allergen-free" antirabies vaccine prepared from the brain tissue of newborn rats. The research done by Kabat et al. (1946, 1947) and by Svet-Moldavskij and his associates (1959, 1960, 1962, 1965) served as a basis for this technique. Independently of these studies in the USSR, the production of an allergen-free antirabies vaccine from the brains of suckling animals has been described by other workers (Fuenzalida et al., 1964; Gispen et al., 1965).

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[2] Head, Laboratory for Vaccine Lyophilization, Moscow Institute for Research on Virus Preparations, Moscow, USSR

Preparation of the Vaccine

Suckling rats aged 4–8 days are used for preparing the lyophilized antirabies vaccine. The Pasteur strain of fixed virus, which has undergone 3249 passages through rabbit brain, is used for infection. A sterile suspension of the brain of an infected rabbit is administered to the rats intracerebrally in a dose of 0.03 ml and a dilution of 1 : 100. After infection, the rats are put back in the cages with the nursing females. Some 70–74 hours later, animals showing symptoms of rabies are killed with ether. The skull is opened under sterile conditions and the brain is removed and placed in a test-tube with 3 ml of a 1% solution of phenol in distilled water.

A piece of cerebellum is placed in sugar broth for a sterility test. Until this test is completed, the brains are kept at a temperature of 4°C. The sterile brain tissue is then homogenized and a 1% solution of phenol in distilled water is added to the homogenate until a 13.3% brain suspension is obtained. This suspension is placed in the incubator for 14 days at a constant temperature of 22°C in order to inactivate the virus. During the whole period of inactivation the suspension is stirred daily.

The initial concentration of virus in the brain suspension is quite high (10^{-6}–10^{-7} LD_{50} per 0.03 ml), which demonstrates that it multiplies intensively in the brain of newborn rats. The reduction in infectivity of the brain suspension proceeds quite quickly during the first 3 days of inactivation and then slowly up to the 14th day.

Freeze-Drying

After inactivation the suspension is diluted with a 30% solution of sucrose in distilled water until it contains 10% of brain suspension and 7.5% of sucrose. This vaccine is dispensed in 1.5-ml quantities into ampoules and is then frozen at a temperature of −45° to −50°C and quickly placed in the chamber of a vacuum-drying apparatus.

Lyophilization of the vaccine is carried out for 26–29 hours, decreasing the pressure gradually from 0.01 to 0.003 mm Hg. Between 6 and 18 hours after the beginning of freeze-drying the heating element is switched on to heat the freeze-dried material to 26–28°C and this temperature is maintained until the end of the process.

After lyophilization the ampoules are flame-sealed, care being taken to preserve the vacuum. The residual moisture content of the lyophilized vaccine is determined by heating weighed portions of 100–150 mg at 100°C for 1 hour in vessels of 7–8 ml capacity. The mean of 2 or 3 determinations should be less than 3%.

Tests

The dried vaccine should be tested for sterility, safety, infectivity, and immunogenicity in the same way as liquid vaccines (see chapter 19, pages 196–

198). For this purpose the contents of an ampoule are reconstituted in 3 ml of sterile physiological saline.

Expiry Date

The activity of the freeze-dried vaccine is unchanged for a period of 6 months if kept at 37°C. When stored at 4°C, as recommended, the expiry date is 18 months after the time of manufacture.

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CHAPTER 23

SUCKLING MOUSE BRAIN VACCINE

E. FUENZALIDA [1]

The suckling mouse brain vaccine (SMB) was developed at the Bacteriological Institute of Chile in 1954 (Fuenzalida & Palacios, 1955). This vaccine was originally used in dogs ; use in man was begun experimentally in 1960 (Fuenzalida et al., 1964). In the period 1960–1962, 2400 controlled treatments were carried out. Since that time SMB vaccine has been the only one administered to man and dogs in Chile (Fuenzalida et al., 1966 ; Fuenzalida, 1967). It is also used in most other South American countries and in Mexico for the prophylaxis of human rabies. In Argentina, Chile, and Uruguay it is the commonly used vaccine for dogs in rabies control programmes.

Formula of the Vaccine

Each 2-ml dose for human use contains the inactivated virus in 20 mg of suckling mouse brain, phenol in a concentration of 1 : 1000, and thiomersal [2] in a concentration of 1 : 10 000.

Each 2-ml dose for use in dogs contains inactivated virus in 50 mg of suckling mouse brain with the same preservatives.

Preparation of Inoculum

Virus strains

Three strains of fixed rabies virus are used for the preparation of this vaccine : 2 strains isolated in Chile (strains 51 and 91, of dog and human origin respectively) and the CVS strain.[3]

Strains 51 and 91 have been maintained since their isolation by intracerebral passages in adult mice (Fuenzalida & Palacios, 1955).

Seed preparation

"Seed stocks" are prepared from 20% suspensions of brains obtained from 3–4-week old mice, previously inoculated with the respective strains.

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[1] Pan American Zoonoses Center, Pan American Health Organization, Ramos Mejía, Buenos Aires, Argentina.

[2] Also known as thimerosal and merthiolate.

[3] The WHO Expert Committee on Rabies, in its sixth report (1973), stresses the need to use only well characterized virus strains for vaccine production. Preferably, only a single strain should be used. — ED.

These suspensions are lyophilized in 0.5-ml volumes and kept at −20°C for a period up to 5 years.

Preparation of the " working stock suspension "

Adult mouse brains infected with the seed of each strain are used to prepare a 20% suspension (w/v) in 2% horse serum in double-distilled water containing 200 IU of penicillin and 200 µg of streptomycin per ml. The suspension is stored in vials at −30° to −70°C and forms the "working stock suspension" from which dilutions are prepared for mouse inoculation, as described below.

Test for viral contaminants

To determine whether the "working stock suspension" is contaminated with neurotropic virus other than rabies virus, 0.5 ml of the supernatant liquid from a 1 : 10 dilution of this suspension is incubated for 90 minutes at 37°C with 0.5 ml of undiluted antirabies hyperimmune serum. The serum is prepared in horses or rabbits immunized with virus grown in animals other than mice. The mixture is inoculated intracerebrally in doses of 0.01 ml into 8 suckling mice and in doses of 0.03 ml into 3–4-week old mice. All the inoculated animals must remain well for 30 days.

Inoculum

To prepare the inoculum, the supernatant liquid from the "working stock suspension", which must be sterile, is diluted until it contains about 100 LD_{50}.

Inoculation and Harvest

Suckling mice *not older than 4 days* are inoculated intracerebrally with 0.01 ml of the inoculum. The inoculum is also injected intracerebrally in doses of 0.03 ml into 10 mice weighing 11–14 g each to determine the viability of the virus. Within 14 days after inoculation all mice must develop signs of rabies. If some mice remain healthy during this period, the "working stock suspension" must be renewed, but the harvest can be used ; if none becomes ill, the mice inoculated for harvest are discarded.

Approximately 96 hours after inoculation, i.e., a day before the usual end of the incubation period, all mice are killed using ether or chloroform. Harvest at this time avoids loss from early deaths and ensures that the virus titre is at maximum.

The killed mice are secured to a dissection board by means of a rubber band. The skin of the head and neck of each animal is disinfected with tincture of iodine or any other adequate disinfectant, or the animals are

(1–1½-in, 15-gauge) needle. The needle is inserted into the cranial cavity in the frontal area. The brain material is aspirated gently, without lateral movements, and expressed into a flask of more than 100-ml capacity. The flask should have a screw cap. During harvest the bottom of the flask is submerged in a mixture of crushed ice and salt. If the collected material is not to be used immediately, it is stored at −20° to −30°C. The average weight of a single brain obtained in this way varies from 0.20 g to 0.25 g.

For large-scale production it is convenient to use a vacuum pump connected to a thick-walled flask by means of a rubber hose (internal diameter 5 mm) with a cotton filter. The needle (40 mm × 1.8 mm; 1½-in, 15-gauge) is connected to the flask by another tube that extends about 3 cm into the flask. All mice are placed in a gauze package which is submerged for 10 minutes in 5% phenol. These mice are put on a tray inside the harvest chamber and are held with gloved forefinger and thumb while introducing the needle. The vacuum is regulated by means of a Mohr clamp.

Preparation of the Vaccine

Immediately before preparation of the vaccine, the brains are partially thawed at room temperature. A 10% suspension (w/v) of tissue in cold, double-distilled water is prepared. To obtain a homogeneous suspension, a blender or some similar apparatus is used and 3 runs of 1 minute each are made, allowing intervals of 10–20 seconds between the runs.

When vaccine is prepared for use in man, the 10% tissue suspension is centrifuged for 10 minutes at 710–2150 *g* and the supernatant liquid is used. Sufficient cold, double-distilled water is added to restore the original volume.

Virus titration

The virus titre of the supernatant liquid is determined by intracerebral inoculation of serial tenfold dilutions into mice (4–5 weeks old, 11–14 g). It is advisable to perform this titration as soon as possible. Titres ranging from $10^{6.3}$ to $10^{7.3}$ are considered acceptable.

Test for viral contaminants

This test is done in the same way as for the "working stock suspension" (see page 217).

Test for bacterial contaminants

Before inactivation by irradiation with ultraviolet light, a titration is performed to detect bacterial contamination. Each of 5 tubes containing

9 ml of thioglycolate broth is inoculated with 1 ml of the whole suspension. One tube is agitated manually and 1 ml of the suspension is transferred to each of a second series of 5 tubes. This procedure is continued to the 10^{-6} dilution. All tubes are incubated and observed daily for 7 days. Bacterial growth must not occur beyond the 10^{-2} dilution in vaccines intended for human use. Although the bacteria are destroyed by ultraviolet irradiation, a high degree of contamination indicates deficiencies in technique and increases local reactions.

Inactivation

A twofold dilution of the 10% suspension is prepared by adding cold, sterile, double-distilled water to obtain a 5% concentration. The virus is inactivated by ultraviolet light using a Dill continuous flow plasma sterilizer (see chapter 25). The material is exposed to a set of 4 ultraviolet lamps (Westinghouse, model G36T6L) and is processed at a rate of about 250 ml per minute.

Safety test

Ten mice, each weighing 18–20 g, are inoculated intracerebrally with 0.03 ml of the irradiated bulk vaccine. Two rabbits, each weighing 1.5–2.5 kg, are inoculated intracerebrally with 0.25 ml of the irradiated bulk vaccine. All test animals must remain free from rabies symptoms and from other diseases of the central nervous system. The observation period is 2 weeks at least.

Final dilution of the vaccine

Immediately after irradiation, the 5% suspension is diluted by the addition of 4 volumes of double-distilled water containing sufficient phenol and thiomersal to achieve final concentrations of 1 : 1000 and 1 : 10 000 respectively. If the vaccine is to be used in dogs, the 5% suspension must be diluted in 1 volume of double-distilled water containing the same preservatives in correspondingly greater concentrations.

Phenol and thiomersal are used to protect the antigen against the action of enzymes in the nervous tissue as well as to preserve sterility.

Sterility test on bulk vaccine

All flasks containing vaccine must be tested for sterility. One ml from each flask is inoculated into at least 4 tubes of thioglycolate broth and 4 tubes of Sabouraud's agar. The tubes are incubated for 7 days, the former group at 37°C and the latter group at 22°C.

The product should not be dispensed into vials until all the above

Immunogenic potency is determined by the Habel test (chapter 31) or, if an appropriate reference standard is available, by the NIH test (chapter 33). When the Habel test is performed, the vaccine is diluted to a final concentration of 0.1%.

Sterility tests on final container

Once the product has been dispensed, sterility tests are conducted on a representative number of flasks or vials of each batch of vaccine.

As a safety measure it is essential that the producing laboratory should preserve the following samples of each batch of vaccine until 2 months after the expiry date for possible future reference :

30 ml of 10% virulent suspension frozen at −20°C or −70°C;
30 ml of 5% inactivated suspension frozen at −20°C;
30 ml of refrigerated vaccine at −4°C or −7°C.

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SUCKLING RABBIT BRAIN VACCINE

R. GISPEN[1]

Formula

The vaccine is an ultraviolet-light-irradiated 5% aqueous suspension of suckling rabbit brain inoculated with fixed rabies virus not later than 24 hours after birth. The suspension contains 2% of McIlvain buffer (pH 7.2) and 5% of lactose and is lyophilized in quantities of one dose, i.e., 2 ml. The vaccine is reconstituted in 2 ml of a 0.01% solution of thiomersal[2] in distilled water per ampoule.

Virus

The Pasteur strain of fixed rabies virus is used. The strain has been passed once every 2 months through adult rabbit brain. Infected brain is kept in 70% glycerol at −15°C.

Rabbits from a controlled nursery stock are inoculated intracerebrally with 0.25 ml of a 0.1% suspension of infected adult rabbit brain in a 2% solution of normal horse serum. The animals are bled to death on the 6th or 7th day after inoculation when paralysis is complete. The brains are removed. Small samples are taken from the tissue for sterility tests. The brains are placed within a few minutes in 70% sterile glycerol at −15°C.

Inoculum

The inoculum is made from the last stock virus. The brain is washed in sterile distilled water and emulsified in a sterile mortar to obtain a 10% suspension in a 2% solution of horse serum in distilled water. The 10% suspension is tested for sterility, placed in small volumes in airtight containers at −70°C, and used as seed virus. A fresh inoculum is prepared on each inoculation day by diluting the 10% suspension to a final tissue concentration of 10^{-3} (w/v) in 2% horse serum.

Inoculation

Newborn rabbits from a controlled nursery stock are used. The animals are kept apart from the mothers in a ventilated incubator at a temperature mating. The suckling rabbits are fed orally through a pipette with a mixture of 7 parts of milk and 10 parts of fresh whole egg. About 6 ml of the food are given twice a day. When the food is refused a subcutaneous injection of 3% dextrose in physiological salt solution should be given.

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[1] Director, National Institute of Public Health, Utrecht-Bilthoven, Netherlands.

The rabbits are inoculated with fixed virus at the earliest convenient time, but not later than 24 hours after birth. The skin on the skull of the animal is painted with a 3% tincture of iodine and 0.15 ml of the inoculum is introduced through the skull about midway between eye and ear, using a 0.90-mm (20-gauge) needle.

Harvest

The infected rabbits show the first signs of paralysis 3 days after inoculation (Fig. 1). On the 4th day, or the 5th day at the latest, the paralysed rabbits are killed by carbon dioxide gas. Animals found dead are not used.

The rabbit is fastened and swabbed with tincture of iodine. The skin on the skull is removed. The exposed skull is painted with tincture of iodine and cut away with sterile scissors. Each brain is transferred into a separate small sterile bottle. A small piece of each brain is tested bacteriologically for sterility. The harvested brains are placed in a refrigerator at —20°C without delay. Contaminated brains are discarded.

Preparation of Vaccine

Brains are placed in a weighed sterile bottle with glass beads. The bottle is weighed again and the weight of the pooled brains is calculated. After the brains have thawed, the bottle is wrapped in cotton wool, placed in a metal container, and shaken by hand for a few minutes. Two parts of a precooled 2% McIlvain buffer solution (pH 7.2) in distilled water are added (see *Diluent* below), and the suspension is homogenized in a mechanical shaker for 1 hour.

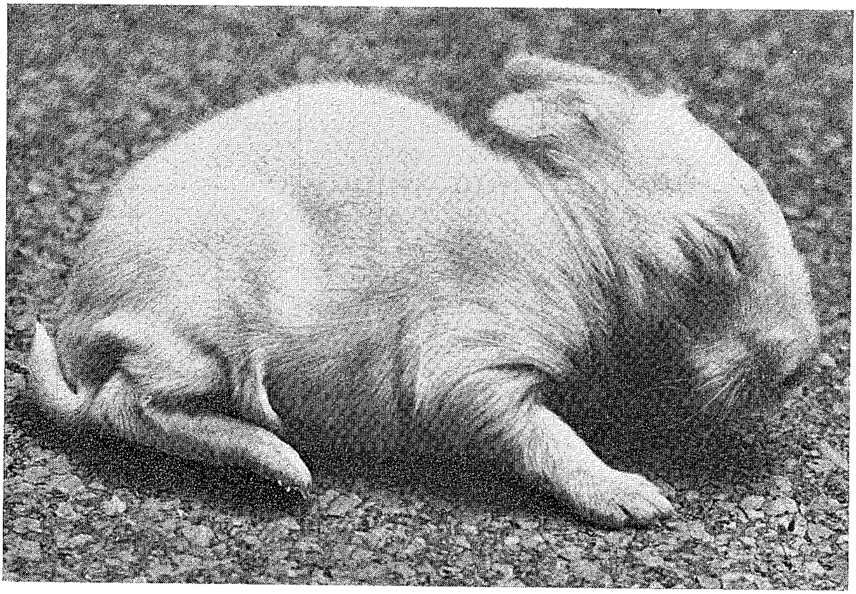
The suspension is further diluted to a 10% tissue suspension (w/v) by adding 7 parts of precooled 2% McIlvain buffer solution (pH 7.2).

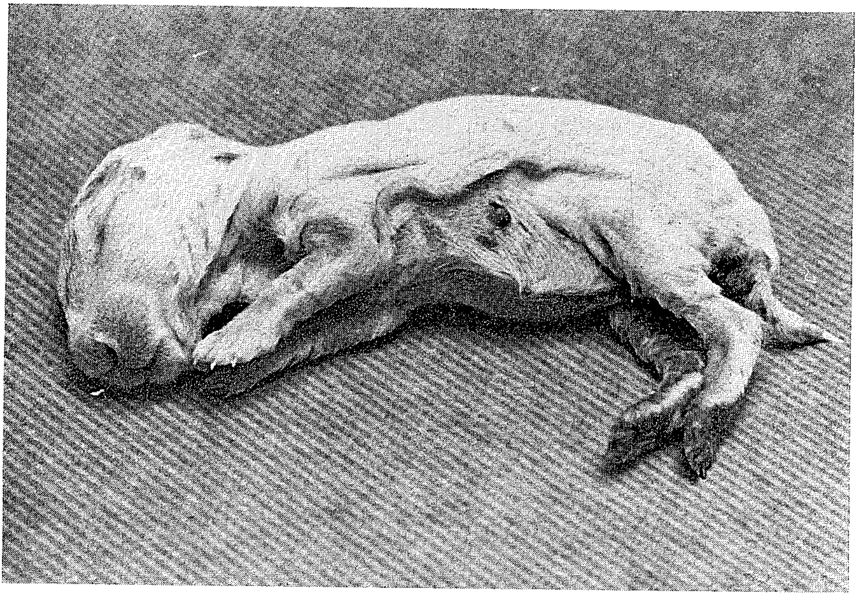
The 10% suspension is titrated by inoculating mice intracerebrally with 0.03 ml (see *Virus titration* below). Another sample of the 10% suspension is used for an identity test (see below).

An equal volume of 10% lactose in 2% McIlvain buffer solution is added to the 10% brain tissue suspension. The suspension containing 5% brain tissue and 5% lactose in buffer solution is passed through a gauze filter to remove unground particles.

The vaccine is inactivated by irradiation with ultraviolet light in a Dill apparatus (see chapter 25). The rate of flow used is 300 ml of vaccine per minute.

FIG. 1. PARALYSED SUCKLING RABBIT AND CONTROL

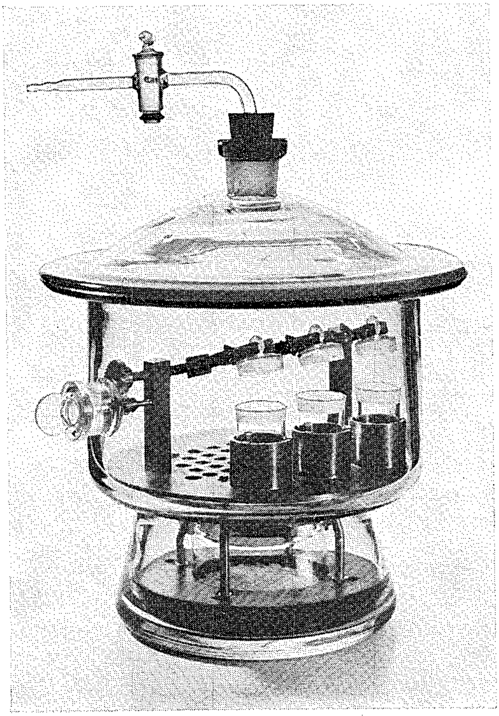




tests. The bulk vaccine is filled into ampoules in 2-ml quantities and frozen at −25°C on the day of irradiation. During the filling process the vaccine should be kept stirred. The vaccine is dried *in vacuo* from the frozen state.

The ampoules are flame-sealed under vacuum. Each ampoule is vacuum-tested. The residual moisture in the lyophilized vaccine should be less than 2%, as determined in portions of 80–100 mg by weighing and drying over phosphorus pentoxide *in vacuo* at 56°C until the weight is constant. To avoid errors in determining the dry weight, atmospheric pressure is restored by admitting dry air and a special device is used that permits the weighing-bottles to be closed before the desiccator is opened (Fig. 2).

FIG. 2. DEVICE FOR CLOSING WEIGHING-BOTTLES INSIDE A DESICCATOR



Diluent (Mc Ilvain buffer, 2%)

Solution A : Dissolve 21 g of analytical grade citric acid ($C_6H_8O_7,H_2O$) in 1000 ml of distilled water.

Solution B: Dissolve 35.6 g of analytical grade sodium hydrogen phosphate (Na_2HPO_4, $2H_2O$) in 1000 ml of distilled water.

Mix 17.65 ml of solution A and 82.35 ml of solution B. One part of this mixture is added to 49 parts of distilled water to make 2% McIlvain buffer solution. The 2% solution is heated for 1 hour at 100°C. The final pH is 7.2. All distilled water is boiled for 10 minutes and cooled before use.

Testing of Virus Suspension

Identity test

A mixture of equal quantities of a 1 : 10^4 dilution of virus suspension and a 1 : 10 dilution of inactivated antirabies serum[1] is incubated in a water-bath at 37°C for 1 hour. Five mice weighing 11–15 g are inoculated intracerebrally with 0.03 ml of the mixture. Another group of mice is treated similarly with a mixture prepared with normal serum. All mice in the latter group should die, whereas at least 4 out of the 5 mice in the specific serum group should survive.

Virus titration

The virus suspension before irradiation is diluted 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} in 2% inactivated normal horse serum in distilled water. For each dilution, 10 mice of 11–15 g are inoculated intracerebrally with 0.03 ml. The 50% endpoint dilution should correspond to at least 10^{-6} LD_{50} per 0.03 ml.

Testing of Irradiated Bulk Vaccine

Sterility test

A representative sample of 30 ml is tested. A few drops are inoculated into each of 2 tubes containing Tarozzi medium[2]. Each of 6 large tubes containing 35 ml of fluid Sabouraud medium[3] are inoculated with 1 ml, and one of these tubes is inoculated with 50 colony-forming units of *Candida albicans* in addition. Five ml of the bulk vaccine are inoculated into each of 2 flasks containing 200 ml of fluid thioglycolate medium.

The vaccine is incubated at 37°C for 7 days with Tarozzi medium, at 18–25°C for 14 days with Sabouraud medium, and at 30°C for 7 days with

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[1] Obtainable from the Pasteur Institute, Paris, France.

[2] Ground beef liver, 500 g; peptone, 10 g; dipotassium phosphate, 1 g; distilled water, to make 1000 ml. Final pH: 9.0.

Candida albicans and absent in all the test containers. If the turbidity of the inoculum interferes with reading, the test medium is mixed after the incubation period and a sample from it is inoculated into sterile medium and tested in a similar way.

Safety test

Ten mice, each weighing 18–20 g, are inoculated intracerebrally with 0.03 ml of the irradiated bulk vaccine. Two rabbits, each weighing 1.5–2.5 kg, are inoculated intracerebrally with 0.25 ml of the irradiated bulk vaccine. All test animals must remain free from rabies symptoms and from other diseases of the central nervous system. The observation period is 2 weeks at least.

Testing of Lyophilized Vaccine

Sterility test

Each filling of the lyophilized vaccine in its final containers is tested. At least 10 final containers are collected as samples. The vaccine in each container is reconstituted by the addition of 2 ml of sterile water.

From each of these containers, 1 ml is inoculated into 40 ml of thioglycolate medium, a few drops are inoculated into a tube of Tarozzi medium, and the remainder is inoculated into 35 ml of fluid Sabouraud medium. For the incubation and reading procedures, see under *Testing of irradiated bulk vaccine* above.

The vaccine in the final container should be proved sterile.

Innocuity test

Four mice weighing about 20 g each are inoculated intraperitoneally with 0.5 ml of the reconstituted vaccine.

Two guineapigs weighing about 350 g are inoculated with 5 ml of the reconstituted vaccine.

All animals should be free from symptoms and survive for 7 days.

Test for encephalitogenic activity

See chapter 36.

Potency test

The potency is tested by the NIH method (see chapter 33).

Reconstitution and Dose

The lyophilized vaccine is reconstituted in 2 ml of 0.01% thiomersal in distilled water. Fourteen daily doses of 2 ml of the reconstituted vaccine, containing 5% (w/v) brain tissue, are recommended for post-exposure treatment.

Storage

The lyophilized vaccine should be stored by the manufacturer at −10°C and may be released not later than 18 months after the date of the last satisfactory potency test.

The expiry date is 6 months after the date of issue, provided that the vaccine has been stored at a temperature below 6°C.

Serological Response

It is recommended that the antibody response to full treatment with 14 daily doses in man be tested by a neutralization test or by a quantitative indirect immunofluorescence test. A significant response should be obtained in all patients treated (Gispen & Saathof, 1965).

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ULTRAVIOLET-LIGHT IRRADIATION FOR INACTIVATION OF VACCINES

K. HABEL [1]

Principles and Methods

The use of ultraviolet (UV) energy to inactivate micro-organisms without impairing their antigenicity is a well established procedure.

UV energy has little ability to penetrate biological substances since it is absorbed rapidly. Therefore, for effective exposure, the material being irradiated must be presented to the incident UV rays in a very thin film. The use of UV-irradiated rabies vaccine became a practical possibility only after the development of apparatus that would accomplish this thin-film type of exposure. It is also well known that as the amount of UV energy absorbed by biological material increases, the degree of chemical change in that material increases ; the length of exposure must therefore be as short as possible while accomplishing the purpose of inactivation, otherwise breakdown of the antigen will occur. The biological effect of UV irradiation on viruses is, for all practical purposes, instantaneous and once this immediate effect has taken place, no further action ensues. There is no evidence that any secondary byproducts that would subsequently be deleterious to the antigen are formed in the irradiation process.

Besides a controlled length of exposure and irradiation of the material in a thin film, for the practical production of large volumes of vaccine it is necessary that the apparatus used should allow for a continuous flow of the antigen suspension through the equipment. Although quartz is a good conductor of UV energy and has been used extensively for making various types of container in which an antigen could be exposed, in general an apparatus designed to eliminate the passage of the UV rays through quartz gives more efficient radiation. Ease of operation and the use of a readily available, inexpensive source of UV are, of course, other desirable features of any practical irradiation apparatus.

Apparatus

Several types of apparatus have been designed that in some way incorporate the principles listed above. One type that is available commercially in the USA and has been proved to be satisfactory for the production of potent rabies vaccines is the Dill apparatus.[1] This employs a standard high-energy germicidal lamp that has a long effective life and is readily available and relatively inexpensive.

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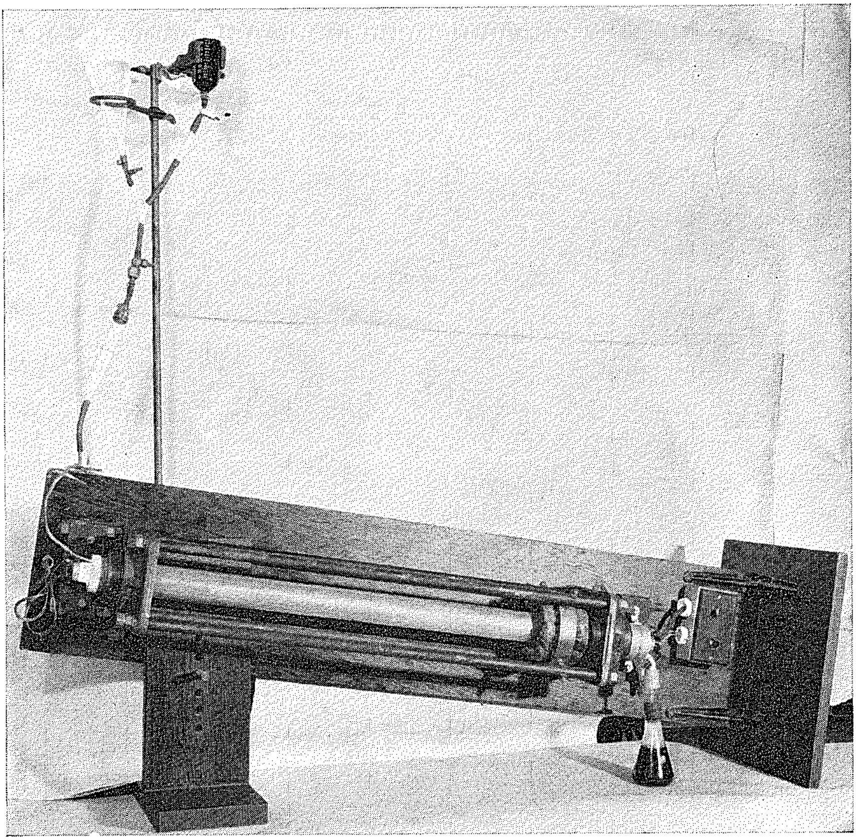
[1] *Formerly* Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif., USA.

FIG. 1. CONTINUOUS-FLOW UV-EXPOSURE APPARATUS



The construction of the type of apparatus of which the Dill machine is a modification can be accomplished by any modern machine-shop from basic materials that are readily available and not excessively expensive. The shop-made model used in our laboratory consists of a long hollow metal or glass tube, preferably of stainless steel, so that the inner surface over which the virus suspension flows is very smooth and easily cleaned. This tube is of such dimensions that a germicidal lamp suspended

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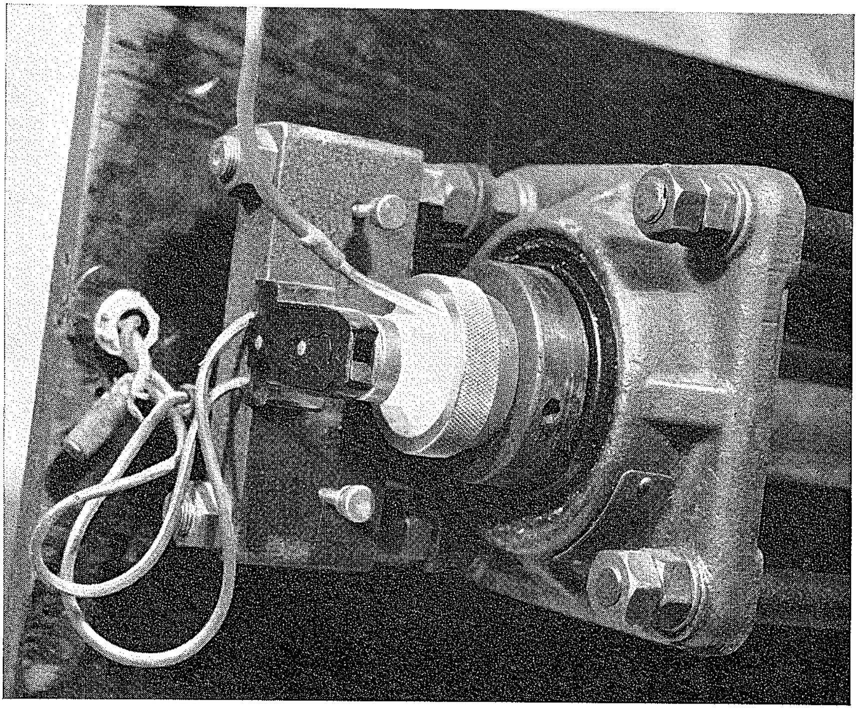
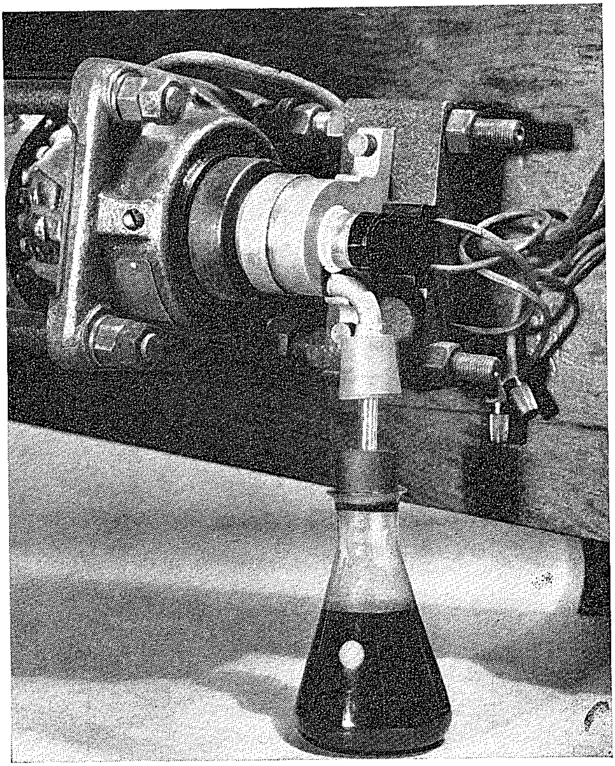
inner wall. This tube is fitted to slide smoothly into an outer, more sturdy, tube made of brass, which is mounted in bearings at each end. These tubes are to be rotated at a speed corresponding to about 150 *g*, so a driving force must be applied. This may be accomplished by a gear or belt drive, but in our own apparatus it is achieved rather simply by making the outer brass tube the armature of a small AC motor of the type used in electric fans. For long runs in large-scale production this may result in greater heating of the tube.

FIG. 2 INTAKE END OF UV-EXPOSURE APPARATUS



A suitably designed collecting-cup fits tightly to the discharge end of the rotating tube for collecting the vaccine as it flows through the apparatus. A supporting frame holds the rotating tube at an angle of 20° and also serves as the place of attachment for the sockets of the UV lamp. The virus suspension is introduced through a 1.6-mm (16-gauge) needle against the inner side of the rotating inside tube, where it is immediately spread in a film approximately 1 mm in thickness which progresses the length of the tube. The film is thus continuously exposed to UV light and is collected through a closed connexion into a flask at the opposite end. A burette or inverted flask may be used as the original reservoir of virus suspension, and is connected to the intake needle by rubber tubing, with a valve for adjusting the rate of flow inserted in the line (see Fig. 1–3).

FIG. 3. COLLECTING END OF UV-EXPOSURE APPARATUS



Some difficulty is occasionally experienced in maintaining a uniform flow rate when the flow by gravity is regulated by a valve in the line. This can be overcome by the use of a finger pump in which the solution is "milked" through the tubing at a constant rate.

The lamp used in this particular apparatus is of a germicidal type, 30-watt, and 91.5 cm (36 in) in length.

With the rates of flow necessary for the proper exposure in this type of apparatus, there is little opportunity for the heat from the UV lamp to be absorbed by the flowing emulsion. Actual thermocouple measurements show that the temperature of the emulsion increases by only 1–2°C in the usual run. However, where large batches (over 500 ml) are irradiated

From the practical standpoint the only standardization necessary is the determination of the minimum exposure (or maximum rate of flow) necessary to inactivate the standard rabies-virus emulsion for routine use in vaccine production. In the apparatus described, a single run with the following rates of flow should give a range of exposure from one completely inactivating the virus to one in which virus is still viable :

50 ml per minute
100 ml per minute
200 ml per minute
400 ml per minute.

The actual exposure time may be calculated after determining the volume of material in the rotating tube at any one time. A simple method of determining this volume is by the introduction of a dye into the intake end of the tube while the test emulsion is being run. Collection of the sample should be started at the exact time the dye is introduced and stopped on its first appearance at the collecting end. The volume of this sample is the approximate amount in the rotating tube. When this volume, the total volume of any run, and the total time of that run, are known, the following formula will give the exposure time :

$$\frac{\text{tube volume (ml)} \times \text{total time (minutes)}}{\text{total volume (ml)}} = \text{exposure time (minutes).}$$

Once the minimum exposure regularly causing virus inactivation has been established, the rate of flow should be set so that, for routine production, twice this amount of exposure will take place. This gives a satisfactory safety factor beyond the point of bare inactivation of the virus, and yet well within the limits of exposure possible before destruction of the antigen with loss of immunizing potency. It has been found that 5 times the minimum inactivating exposure is necessary before any marked drop in antigenic potency occurs.

Under ideal operating conditions there should be a continuous monitoring of the amount of UV energy to which the emulsion is being exposed.

Virus Emulsion

All the factors of importance in rabies-vaccine production by any other method of inactivation are of equal importance for material to be irradiated. The virus titre of the original emulsion should be as high as possible, but variation of as much as one in the log titre will not noticeably change the necessary exposure time. Since UV light will not penetrate biological materials to any appreciable depth, it is quite important that the emulsion be a uniformly fine one. To ensure this it is desirable to screen the emulsion through several layers of sterile surgical gauze or a wire screen.

In general, a 5% whole emulsion of infected nervous tissue is used with the irradiation equipment, but no difficulty is encountered in inactivating suspensions up to 10% in concentration. Virus suspension from sources other than brain emulsions can also be inactivated in this apparatus.

Preservation and storage of irradiated vaccine

If irradiated vaccine is to be kept in the liquid state before use, a chemical preservative should be added. Thiomersal[1] at a 1 : 8000 dilution, or 0.25% phenol, appears to be superior to formaldehyde. Irradiated vaccine, because it contains no substance in itself deleterious to viral antigen, may be kept frozen or may be dried from the frozen state. Freeze-drying will cause an immediate drop in immunizing potency, but the vaccine will then hold that potency indefinitely if sufficiently dry.

Testing of irradiated vaccine

Sterility, safety, and potency tests should be carried out on the irradiated vaccine in the same manner as with other types of inactivated rabies vaccine. Since it is difficult to monitor the effectiveness of the radiation to which the vaccine is being exposed, it is especially important that both potency and safety tests be carried out routinely on every batch of the final product. This is the only way to check whether irradiation has proceeded according to plan.

In general, it has been found that highly potent vaccines can be made consistently by the irradiation method. There is no evidence to date that the incidence of postvaccinal complications is any greater after the use of irradiated vaccine than with other type.

Steps in an irradiation run

(1) The inner tube is cleaned with soap or detergent solution, thoroughly rinsed, and then cleaned with ether.

(2) The apparatus is assembled, and the UV lamp inserted through the tube and clamped into its sockets.

(3) The collecting end-piece, which has been autoclaved, is connected to the apparatus and, through sterilized rubber tubing, to the collecting flask.

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with virus emulsion, are connected by a Y-tube to the rubber tubing going to the intake needle through a flow valve or through a finger pump.

(5) The intake needle is adjusted to direct flow against the inner side of the rotating tube, and is fixed in that position.

(6) The UV lamp is turned on for 5 minutes to sterilize the inside of the rotating tube, the motor of which has been started.

(7) Distilled water is run through the apparatus to wet down the tube, and the flow is adjusted to the desired rate.

(8) The flow of the distilled water is stopped, and that of the virus emulsion started. The rate of flow is rechecked. As soon as the emulsion appears at the collecting end, a fresh collecting flask is attached, and timing of the run started. The time is again taken at the end of the run, and the volume of collected emulsion is measured.

(9) When a series of exposures is being made to determine the maximum flow rate giving inactivation of the virus, the sequence should be from the longest to the shortest exposure.

CHAPTER 26

CHICKEN-EMBRYO VACCINE

H. KOPROWSKI[1]

At present the Flury and the Kelev strains of rabies virus adapted to and modified in the developing chicken embryo are used for the production of chicken-embryo rabies vaccines employed in the prophylactic immunization of dogs.[2] Vaccine made with the Flury strain (Koprowski & Cox, 1948) has been used on a worldwide basis and therefore the description of the production procedure will be mainly confined to the Flury-strain vaccine. The same principles, however, are followed in the preparation of chicken-embryo rabies vaccine with the Kelev strain (Komarov & Horenstein, 1953).

The Flury-strain vaccine for dogs represents the 40th–50th egg-passage level of the virus. At this egg-passage level the Flury virus is fully virulent by the intracerebral route for mice, cotton-rats, hamsters, and guineapigs, while rabbits are more resistant. Hamsters, mice, and cotton-rats are susceptible to extraneural injection, but guineapigs are more resistant. On the other hand, rabbits and dogs fail to show signs of infection when inoculated intramuscularly with concentrated suspensions of the virus.

Further modification of the Flury strain has been achieved by continued egg passage (180th passage), and this has resulted in a lessened pathogenicity for experimental animals while its antigenicity has apparently been retained. For the present, the high egg-passage level vaccine has been recommended for use in cattle and cats and the low egg-passage level vaccine, described here, for use in dogs only.

The Kelev chicken-embryo vaccine represents the 60th–70th egg-passage level of the virus. At this egg-passage level the virus does not produce any signs of infection in hamsters, guineapigs, or rabbits inoculated either extraneurally or intracerebrally. Suckling mice are, however, susceptible when inoculated intracerebrally. Dogs inoculated intramuscularly with concentrated suspensions of virus fail to show signs of infection.

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[1] Director, The Wistar Institute, Philadelphia, Pa., USA.

At the proper egg-passage level of the virus (Flury, 40th–50th ; Kelev, 60th–70th), a sufficient quantity of 60% embryo suspension in distilled water is prepared, tested for potency (see chapter 34, page 287) and preserved either frozen or in its dehydrated form. This constitutes the seed material. Just before use a 20% suspension of seed material in distilled water is prepared and tested for viability as follows.

Inoculate a group of 6 mice intracerebrally with 0.03 ml of a 10^{-1} dilution of the seed material, using a 0.25-ml tuberculin syringe with a 0.40-mm × 6-mm (27-gauge, $\frac{1}{4}$-in) needle. If the seed material contains live virus the mice will sicken on the 6th–8th day. Should inoculated mice remain unaffected by the 9th day, discard the inoculated eggs.

In the case of the Kelev strain the viability test must be carried out in suckling mice.

Inoculation of Eggs

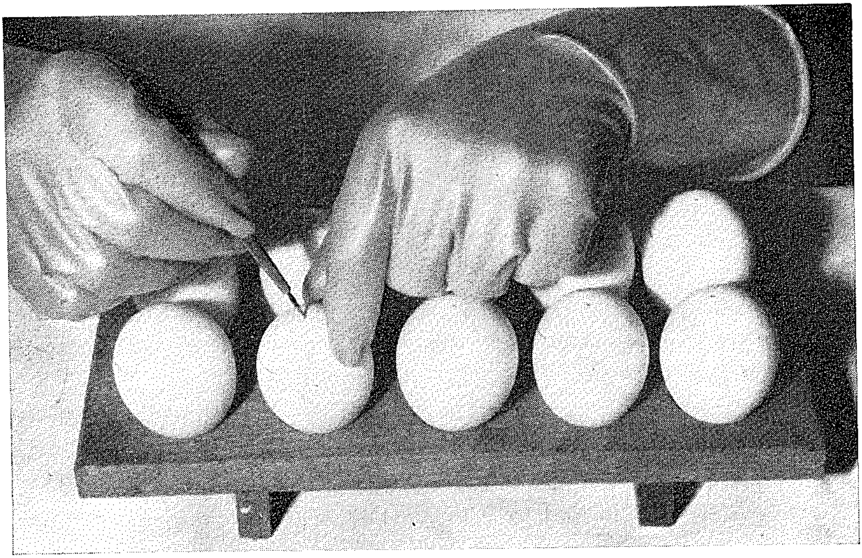
The number of eggs to be inoculated will depend on the amount of vaccine required and on the storage facilities. On a large production scale the average yield is 4–6 dog doses per harvested embryo. Embryo mortality due to non-specific causes does not usually exceed 15%.

Fertile hen's eggs incubated for 7 days are used. It is best to obtain fertile incubated eggs for vaccine production from a commercial hatchery and transfer the eggs to the laboratory one day before inoculation. On the 7th day of incubation the eggs are candled ; unfertilized eggs and those with dead or weak embryos (poor vascularization, sluggishness), and eggs showing displaced air sacs are discarded. While candling, the boundaries of the air sacs are marked on the shell with a pencil and the eggs are arranged air sac uppermost in a tray (a commercial cardboard egg-tray serves the purpose well). The shell is painted at the top of the air sac with 70% alcohol, and flamed briefly.

By means of a carborundum disc attached to a rotary motor tool or a dental drill, make 2 crosswise cuts, or penetrate the shell directly at the top and centre of the air sac. If this type of cutting machine is not available, pierce the shell at the point indicated by means of any sharp instrument (see Fig. 1).

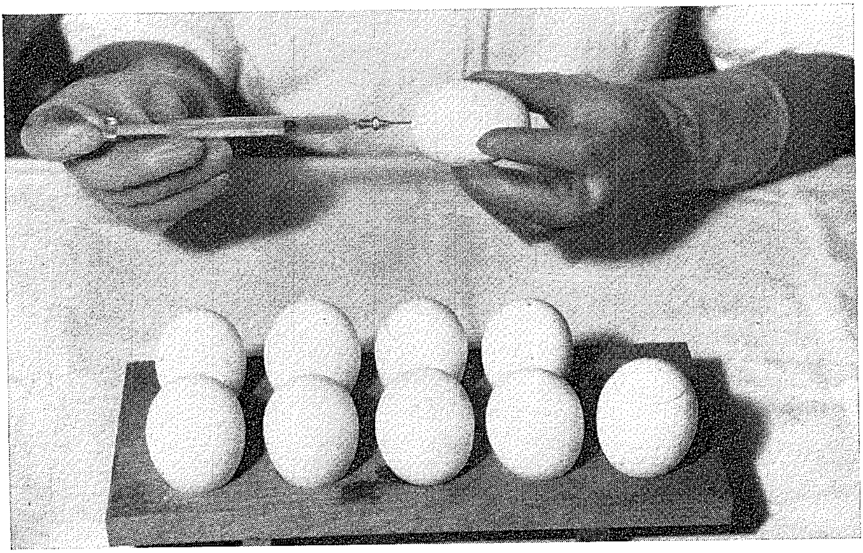
The eggs are inoculated in the yolk sac with 0.25 ml of the 20% suspension of seed material, using a syringe with a 0.90–1.00-mm × 20–25-mm (19–20-gauge, $\frac{3}{4}$–1-in) needle. Fill the syringe with the inoculum and hold it in the right hand parallel to the table ; with the left hand take an egg from the tray and hold it parallel to the table on the same line as the syringe, and with the blunt end of the egg directed toward the syringe. Through the cut in the shell introduce the entire length of the needle into

FIG 1. PIERCING THE AIR SAC



By courtesy of Dr F. Perez-Gallardo, Madrid

FIG. 2. YOLK-SAC INOCULATION OF EGG IN HORIZONTAL POSITION



eggs are replaced in the tray. On completion of the inoculation the seed material is tested for bacterial sterility, and the cut portion of the shell is sealed by means of a melted mixture of paraffin and petroleum jelly (two-thirds paraffin, one-third petroleum jelly), or collodion.

After inoculation the eggs are incubated at 36.5°C for a period of 9–10 days.

Harvest

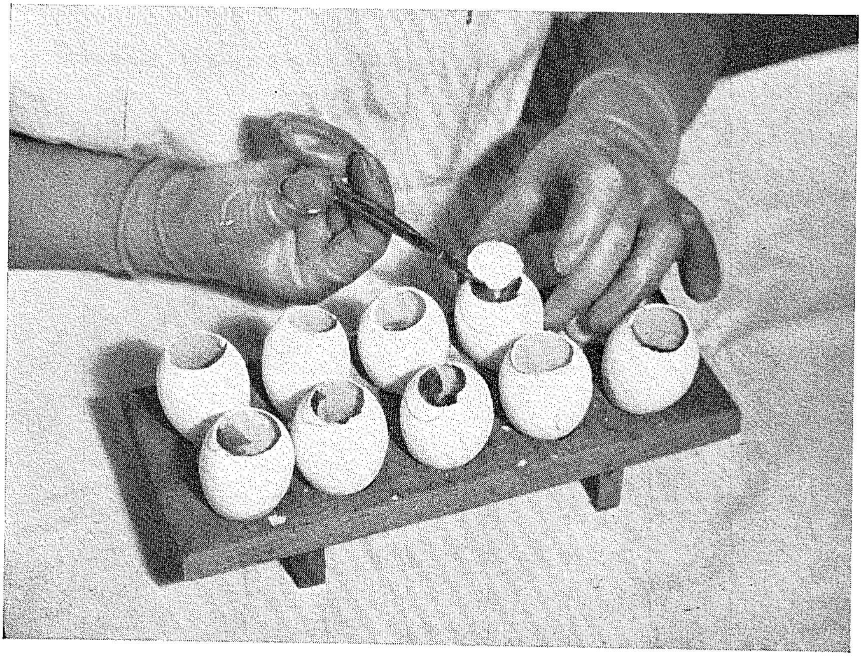
At the end of the incubation period, only living embryos are harvested. Yolk, yolk-sac, extra-embryonic tissue, and fluid are discarded.

The eggs to be harvested are arranged in a tray, and the shells are sprayed or painted with 70% ethanol and flamed.

The live embryos are harvested by one of the following methods:

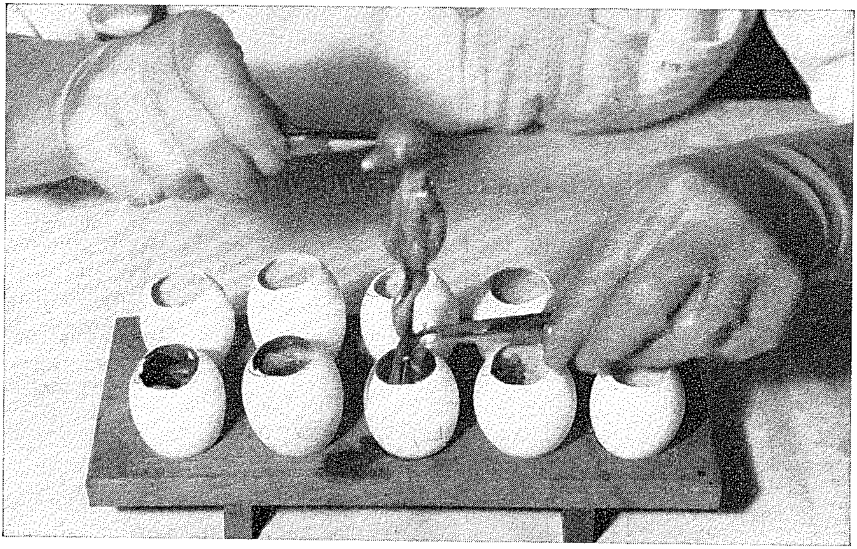
(*a*) Cut off the shell over the air sac by means of sterile sharp-pointed scissors (see Fig. 3). Tear away the membrane, thus exposing the embryo. Pass a wire hook attached to a handle under the neck of the embryo,

FIG. 3. CUTTING OFF SHELL COVERING AIR SAC



By courtesy of Dr F. Perez-Gallardo, Madrid

FIG. 4. SEPARATING EMBRYO FROM MEMBRANES



By courtesy of Dr F. Perez-Gallardo, Madrid

and pull the embryo slowly upward. The embryo will be pulled out of the egg, free from the yolk and extra-embryonic material.

Alternatively, lift the embryo out with one pair of forceps while separating the membranes with another (see Fig. 4).

(*b*) Put on sterile rubber gloves. Crack the shell of the eggs at the centre by means of a sharp sterile instrument. Open the shell with gloved hands and pour the contents over a sterile wire gauze. The fluid and yolk should drop through the meshes into a sterile container placed under the wire gauze layer. Collect the embryos from the layer of gauze into a suitable container.

Preparation of Embryonic Suspension

The harvested embryos are placed in a chilled weighed container. The total weight is noted and then the embryos are transferred to a chilled Waring Blendor. Sufficient chilled sterile distilled water [1] is added to the Blendor to prepare a 66% embryo suspension (5 ml of distilled water for every 10 g of embryos). The embryos are ground 3 or 4 times, each time for about 3 minutes, and the Blendor is chilled between each grinding.

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3 layers of gauze into a chilled container. It is then measured and diluted with an equal volume of a stabilizer[1] to give a final embryo concentration of 33%. The vaccine is placed in ampoules or bottles so that each ampoule or bottle contains 3 ml of 33% suspension, which equals one dog dose.

Lyophilized Vaccine

The embryo emulsion is desiccated from the frozen state. After lyophilization, the ampoules are sealed either in vacuum or in an atmosphere of nitrogen and stored at 4°C. The addition of a stabilizer to the vaccine before lyophilization greatly increases the resistance of the virus to thermal inactivation.

Bacterial Safety Tests

Tests for the presence of pathogenic organisms are made in mice. Eight young adult mice are each inoculated intraperitoneally or subcutaneously with 0.5 ml of the reconstituted vaccine. At least 7 of these mice must remain alive and healthy for a 7-day observation period.

A sample of bulk material collected from each batch before the addition of antibiotics should be tested by classical bacteriological techniques for the presence of *Salmonella*.

Specific Safety Test

A specific safety test on the product is made in at least 2 young dogs. Each dog receives intramuscularly the recommended field dose and is observed for 21 days. For a test to be considered satisfactory, all dogs must remain free of any symptoms that might be attributed to the vaccine. The dogs used in this test should not be used in any subsequent safety test.

Virus Titration in Mice

1. Each of 2 vials of vaccine representing each series or subseries in final containers (irrespective of the manufacturer's recommended dose)

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[1] Preparation of a stabilizer for desiccation of the Flury vaccine (formula adapted from the one used by the Onderstepoort Laboratory, South Africa) :

In one litre of distilled water dissolve :

potassium dihydrogen phosphate . .	2.7 g
peptone (Difco-Bacto)	20.0 g
lactose	100.0 g

This mixture must be heated slightly in order to dissolve the lactose. Cool the mixture to room temperature and adjust the pH to 7.6 with a normal solution of sodium hydroxide (the amount needed depends on the brand of peptone used ; 22 ml are needed for the above brand). Sterilization is obtained by filtration through Seitz or Millipore filters under positive pressure ; do not autoclave.

is reconstituted with sterile distilled water containing 2% of normal horse serum (3 ml of the diluent per dog dose of dried vaccine). Withdraw half a dog dose (1.5 ml) from each of the 2 vials of reconstituted vaccine, or a full dog dose (3 ml) if the 2 vials of reconstituted vaccine are combined, and add this to 7 ml of distilled water containing 2% of normal horse serum in a test tube. This gives 10 ml of diluted vaccine, representing a 10^{-1} dilution.

Antibiotics may be added to the diluent at the discretion of the manufacturer. 500 IU of penicillin and 780 IU (1000 µg) of streptomycin should be added per ml of diluent for each dilution.

2. Using prescribed and acceptable laboratory procedures, make further tenfold dilutions from the 10^{-1} dilution prepared above. A suggested method is as follows:

(*a*) Prepare an appropriate number of test tubes with 4.5 ml of the sterile diluent referred to above.

(*b*) Using a 1-ml pipette, transfer exactly 0.5 ml of the reconstituted, well mixed sample (10^{-1} dilution) to the first tube without touching the diluent with the pipette. Discard the pipette in an appropriate receptacle.

(*c*) Take another 1-ml pipette and mix the suspension in the first tube by drawing and expelling the suspension in and out of the pipette at least 5 and, preferably, 10 times. The mixing should be done as vigorously as possible without causing bubbles to form extensively. Using the same pipette, withdraw enough of the mixture in tube 1 to transfer 0.5 ml to tube 2 without touching the diluent with the pipette. Discard the pipette. Continue this procedure until all the tubes have been used.

(*d*) The reconstituted vaccine (10^{-1} dilution) is mixed with the sterile diluent as prescribed above to give the following dilutions:

Tube No.	*Volume of diluent (ml per dog dose of dried vaccine)*	*Volume transferred (ml)*	*Resultant dilution*
	10		10^{-1}
1	4.5	0.5	10^{-2}
2	4.5	0.5	10^{-3}
3	4.5	0.5	10^{-4}
4	4.5	0.5	10^{-5}
5	4.5	0.5	10^{-6}

Tubes containing the desired dilutions are placed in an ice-bath. The highest dilution to be used is optional; however, the lowest dilution used should kill at least 80% of the mice. At least 10 young adult mice (4–6 weeks of age) approximately 16 g are inoculated intracerebrally with 0.03 ml of each dilution, progressing from the highest to the lowest dilution. At least 80% of the mice inoculated with each dilution must survive and disregarded in determining the percentage that survive the 4-day period.)

Deaths occurring during the first 4 days are not considered in the test. The mice are observed for 14 days, death and paralysis subsequent to the first 4 days being recorded, and the LD_{50} titre is calculated by the Spearman-Kärber or Reed & Muench method (see Appendix 1, page 321). A satisfactory vaccine must have a titre of not less than $10^{3.3}$ LD_{50} per 0.03 ml. This minimum level is to be maintained throughout the expiration period. A suggested minimum titre for release is 0.5 log higher, i.e., $10^{3.8}$ LD_{50} per 0.03 ml.

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CHAPTER 27

DUCK-EMBRYO VACCINE

J. M. HOSKINS[1]

Nature of Vaccine

Duck-embryo rabies vaccine is a lyophilized preparation consisting of duck-embryo tissue and β-propiolactone-inactivated rabies virus. It is stabilized by the incorporation of cysteine hydrochloride, gelatin, lactose, and alkaline potassium phosphate. Streptomycin sulfate and chloramphenicol succinate are present in very low concentration. Thiomersal is added as a preservative. Vaccine for injection is prepared by reconstituting the lyophilized material with sterile distilled water.

Virus strain

The vaccine is prepared with the Pitman-Moore (PM) strain of rabies virus. The PM strain adapted to mouse brain does not grow to sufficiently high titre in the duck embryo for satisfactory routine vaccine production. At the Swiss Serum and Vaccine Institute, Berne, a variant strain of PM virus has been developed by rapid serial passage in young embryonated duck eggs. Virus at the 12th and 13th serial passage level has been selected for use as seed material for vaccine production. At these passage levels the virus grows reproducibly to high titre without significantly affecting embryonic growth or mortality.

Materials and Reagents

Duck eggs

Probably most common breeds of duck can be used as a source of eggs and the choice is likely to be influenced by the commercial availability. It is important to use eggs derived from a healthy flock that is known to be free from *Salmonella* infection. Only naturally clean eggs should be used ; they may, however, be treated with an appropriate disinfectant. For further details see the Annex on p. 252.

Production of vaccine is based on the seed lot system. Primary and secondary seed lots of virus are prepared.[1] For the preparation and testing of seed virus see the Annex on p. 252.

In the method described in this chapter, primary seed virus corresponds to PM virus at the 12th duck-embryo passage level; secondary seed virus corresponds to the 13th passage level. The vaccine is prepared from virus that corresponds to the 14th passage level in duck embryos. Both primary and secondary seed virus consist of a 33% w/v suspension of duck embryo tissue infected with PM virus at the appropriate passage level.

β-Propiolactone

This substance is used to inactivate the bulk vaccine virus suspension before lyophilization. It is an unstable liquid that readily polymerizes upon exposure to elevated temperatures and hydrolyses in the presence of moisture. A preparation that contains a significant amount of polymerized β-propiolactone will precipitate during use and may give rise to irregularities during virus inactivation. The best results are obtained with the freshly distilled compound and it is important that it should be stored in tightly closed containers (preferably in sealed ampoules) at approximately −10°C to −20°C and kept away from light. It should never be stored at temperatures above 0°C or below its freezing point (−33.4°C).[2]

Stabilizing medium

The same stabilizing medium is used for the preparation of both the seed virus and the raw virus suspension for vaccine production. It serves to preserve the infectivity of live virus during freezing and storage and also to stabilize the antigenicity of inactivated virus during lyophilization and subsequent holding of the dried vaccine. The preparation of the stabilizing medium is described in the Annex on p. 253.

Seed virus diluent

For composition and preparation, see Annex.

Thiomersal

This is added to the bulk vaccine virus suspension after inactivation. The preparation of the 1% stock solution is described in the Annex.

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[1] A seed lot is a quantity of virus that is prepared at one time and is uniform with respect to composition. Primary seed virus is virus used to inoculate the substrate (embryonated duck eggs) for the preparation of all secondary seed lots. Secondary seed virus is virus used to inoculate the substrate for the preparation of vaccine. Thus, secondary seed virus is one passage removed from primary seed virus and the virus used for vaccine production is one passage removed from secondary seed virus.

[2] For the method of titration of β-propiolactone, see chapter 21, p. 210.

Inoculation and Harvest of Embryos

Pre-inoculation incubation of eggs

Before inoculation the eggs are incubated for 7 days in a fresh-air incubator maintained at 37.5–38.5°C and 65–75% relative humidity. They should be turned regularly during the entire period of incubation. This is conveniently carried out in an incubator fitted with automatic turning equipment if the eggs are placed in a vertical position and moved through an angle of about 110° once every hour.

Preparation of seed virus for inoculation

An appropriate amount of secondary seed virus is thawed and prepared just before use.

(1) Thaw the seed virus in a water-bath maintained at 36–37°C. Do not warm the thawed suspension to more than about 4°C.

(2) Clarify the virus by centrifuging at approximately 1000 g[1] for 20 minutes at 4°C.

(3) Prepare a 10^{-1} dilution of the supernatant fluid in seed virus diluent. The seed virus is now ready for inoculation.

(4) Place the prepared seed virus in an ice-bath until the actual moment of use.

Inoculation of eggs

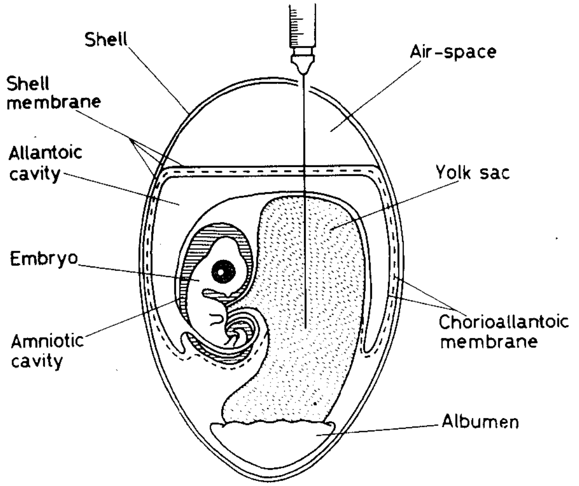
(1) Candle the eggs by transillumination, using a concentrated light source. Retain for inoculation only intact eggs that are normally developed. Discard those that are cracked, infertile, or contain dead embryos. Mark a point on the shell in the centre of the air space.

(2) Disinfect an area approximately 1 cm in diameter over and around the marked point on the shell. Disinfection is done with a swab moistened with tincture of iodine diluted with an equal quantity of 70% ethanol. Take care not to wet the shell more than necessary.

(3) Using an ordinary straight dental drill, make a simple hole in the shell at the marked point. The hole should be no larger than is needed to accommodate comfortably the inoculation needle that is used at the next stage. Do not cut through the underlying shell membrane. The drill may be sterilized by immersing in 70% ethanol for 30 minutes immediately before use.

1-ml automatic syringe of the "Cornwall" type, inoculate 0.25 ml of prepared seed virus directly into the yolk sac. This operation is done blind, by inserting the needle vertically into the egg for almost its entire length (see figure). Care is needed in carrying out the inoculation if a syringe of the "Cornwall" type is used, since this instrument is relatively unwieldy and traumatic damage to the embryo may easily occur. The inoculum should be delivered fairly slowly. Withdraw the needle carefully after inoculation.

EMBRYONATED DUCK EGG: YOLK SAC INOCULATION



(5) Seal the inoculation hole immediately with one or two drops of 4% collodion and leave at room temperature until the collodion solvent has evaporated. Do not transfer eggs that are wet with collodion to the incubator.

Post-inoculation incubation of eggs

After inoculation the eggs are incubated in a fresh-air incubator maintained at 36–37°C and 60–70% relative humidity. They are placed in a vertical position and left undisturbed for 10 days.

Harvest of infected embryos

The embryos are best harvested after incubating for 10 days, although they may be used as early as 7 and as late as 12 days after inoculation. The choice of a 10-day incubation period represents a compromise based upon considerations of economic yield and aesthetic appearance of the final vaccine.

(1) Candle the eggs by transillumination and mark the edge of the air space. At the same time, discard any eggs containing dead embryos.

(2) Disinfect the entire upper surface of the egg, using a swab moistened with tincture of iodine diluted with an equal quantity of 70% ethanol. Take care not to wet the shell more than necessary.

(3) Using a carborundum disc attached to an ordinary dental drill, cut round the edge of the air space, keeping about 1 mm above it. Do not cut through the underlying shell membrane. The carborundum disc may be sterilized by immersing in 70% ethanol for 30 minutes immediately before use.

(4) Remove the shell cap with a scalpel or other sharp instrument and discard.

(5) Using fairly blunt forceps, tear the shell and chorioallantoic membranes and the amniotic sac so that the embryo is made visible. The embryo is then removed intact to a weighed beaker. All other egg materials, such as egg fluids, extra-embryonic membranes, yolk, and albumen are discarded. The beaker in which the embryos are collected is kept in crushed ice until harvesting is complete. Embryos are conveniently collected in groups of up to about 30 individuals (total weight about 250 g).

(6) Reweigh the beaker with the collected embryos and note the weight of the latter.

Homogenization of infected embryos

The infected embryos are homogenized immediately after harvesting. This is conveniently done using a homogenizer of the Waring Blendor type; a one-litre stainless steel blending jar mounted on the standard motor is suitable for the purpose. Alternative homogenizing devices may also be employed, but they should first be shown to be suitable. The main requirement is that the homogenizer should be able to disintegrate the embryos completely, in a sterile condition, without allowing the resulting tissue suspension to become warm. The procedure described below is based upon the Waring Blendor unit.

The embryos are homogenized in a stabilizing medium to produce a 33% w/v tissue suspension. This suspension is then frozen and stored at a temperature lower than —70°C until all control test requirements have been satisfied.

(1) Decant one group of weighed embryos (total weight about 250 g) into a chilled blending jar.

(2) Add sufficient chilled stabilizing medium to make a 33% w/v suspension.

rev/min) and run for 60 seconds only. Wait 1 minute and then run at maximum speed for a further 60 seconds. Wait again for 1 minute and run for a final 60 seconds, also at maximum speed. The embryo homogenate so obtained is designated *raw virus suspension*. After the last run, and without waiting specifically for the contents of the blending jar to settle, decant the raw virus suspension directly into a series of 500-ml blood bottles, or other similar containers. From the last bottle filled, remove samples for assay of virus infectivity and for sterility testing.

(5) Transfer the raw virus suspension direct to a freezer maintained at a temperature lower than −70°C. Freeze and store the sample for virus infectivity assay also under the same conditions. Do not freeze the sample to be used for sterility testing.

Control Tests on Raw Virus Suspension

The tests listed below are carried out according to standard procedures. Consequently, full technical details are not given here.

Assay of virus infectivity

This is done on the raw virus suspension after freezing. It is performed in adult mice, which are inoculated intracerebrally. The titre should be within the range $10^{6.0}$–$10^{6.5}$ LD_{50} per 0.03 ml. However, preparations with a titre of $10^{5.5}$–$10^{6.0}$ LD_{50} per 0.03 ml will yield a vaccine of satisfactory antigenic potency.

Tests for sterility

These tests are performed on the raw virus suspension before freezing. The suspension is not processed further until it has been shown to be bacteriologically and mycologically sterile (see page 225).

Inactivation of the Virus

After appropriate preparation the raw virus suspension is inactivated with β-propiolactone, a substance that reacts readily with hydroxyl, amino, carboxyl, sulfhydryl, and phenolic groups and effectively destroys virus infectivity without affecting antigenicity. In aqueous solution, β-propiolactone is itself rapidly and completely hydrolysed, breaking down into hydracrylic acid. In the presence of aqueous sodium chloride, such as is found in the duck embryo suspension, a certain amount of β-chloropropionic acid is also produced. Both end-products are physiologically harmless, however, so that it is not necessary to remove or neutralize them.

Preparation of raw virus suspension for inactivation

(1) Thaw the raw virus suspension in a water-bath maintained at 36–37°C. Do not warm the thawed suspension to more than about 4°C.

(2) Clarify the virus by centrifuging at approximately 1800 g[1] for 30 minutes at 4°C.

(3) Decant the supernatant fluid into a flask large enough to hold the envisaged total volume. If it is necessary to carry out more than one centrifuge run, the supernatant fluids are best kept in crushed ice until all are collected. The pooled supernatant fluids are designated *bulk vaccine virus suspension*.

Care should be taken when the supernatant fluid is decanted to avoid carrying over significant amounts of retinal pigment. This separates as a dark grey layer above the sediment during centrifugation and tends to float off when the supernatant fluid is decanted. Its inclusion in the final vaccine is avoided only for aesthetic reasons.

(4) Measure the pH of the bulk vaccine virus suspension and adjust it to 7.6 by the addition of 1.0 N sodium hydroxide solution. Approximately 4.5 ml of sodium hydroxide solution are required for each litre of virus suspension.

(5) Measure the total volume of bulk vaccine virus suspension.

(6) Raise the temperature of the bulk vaccine virus suspension to 36–37°C by heating it in a water-bath maintained at the same temperature. The virus suspension is now ready for inactivation.

Inactivation of bulk vaccine virus suspension

Inactivation of the bulk vaccine virus suspension is carried out by incubating it for 2 hours at 36–37°C in the presence of β-propiolactone at an initial concentration of 1 : 2500. However, since β-propiolactone is moderately dense, does not dissolve immediately in water, and once dissolved is rapidly hydrolysed, the method of bringing the 2 reagents together is important. It is achieved by diluting the β-propiolactone in the cold state before adding it to the prewarmed bulk vaccine virus suspension.

(1) Remove an appropriate small amount of β-propiolactone from storage and raise the temperature to about 0°C with crushed ice.

(2) Prepare a 1 : 10 dilution of the cold β-propiolactone in distilled water at the same temperature. This is done by adding the lactone to the water and swirling rapidly to hasten solution. The diluted β-propiolactone

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solution ; it must then be used without delay.

It is important to note that the batch of β-propiolactone will dissolve completely to produce a water-clear solution when diluted in the way just described. If it precipitates on dilution it should not be used.

(3) Add the cold diluted β-propiolactone to the prewarmed bulk vaccine virus suspension and mix immediately.

(4) Immediately after mixing, transfer the entire mixture to a fresh vessel. This step is essential in order to avoid any carry-over into the final vaccine of live virus that may have escaped coming into contact with β-propiolactone by being splashed on the walls of the reaction vessel. It is also necessary to make the transfer while the lactone is still present in relatively high concentration.

The way in which the reaction mixture is transferred depends upon the volume of liquid involved. Volumes up to about 3 litres may be decanted with safety. Larger volumes should be transferred by means of a closed siphon system. Whichever method is employed, care should be taken that the fall in temperature is kept to a minimum.

(5) Allow the mixture to react at 36–37°C for a total of 2 hours from the time when the β-propiolactone is added to the bulk vaccine virus suspension. The mixture is best stirred continuously throughout the reaction period. The product obtained at the end of the reaction period is designated *inactivated virus.*

(6) Cool the inactivated virus rapidly to about 4°C by agitating it in a bath of ice slurry. When cold, transfer it to a refrigerator maintained at approximately 4°C and leave overnight.

(7) Measure the pH of the inactivated virus after overnight refrigeration. It should be within the range 7.0–7.1. The pH is not adjusted.

(8) Remove samples of inactivated virus for testing for the absence of live virus and for sterility. The samples must not be frozen.

(9) Finally, add thiomersal to the inactivated virus to give a final concentration of 1 : 10 000.

Control Tests on Inactivated Virus

The tests listed below are performed as described in chapter 19, pages 196–198. They are done on inactivated virus that has not been frozen.

Test for absence of live virus

No live virus should be demonstrable.

Tests for sterility

The inactivated virus must be bacteriologically and mycologically sterile (see page 225).

Lyophilization

Lyophilization of the thiomersal-treated inactivated virus represents the last stage in vaccine production. It is essential in order to preserve the antigenicity of the vaccine during storage and subsequent distribution. Lyophilization is carried out in single-dose containers.

The lyophilization process is started on the day following inactivation of the bulk vaccine virus suspension, i.e., after the addition of thiomersal to the inactivated virus.

(1) Dispense the thiomersal-treated inactivated virus into 3-ml vials in 1.0-ml amounts. The vials should be of neutral glass with high resistance to thermal and mechanical shock.

(2) Lyophilize the vial contents by freezing at a temperature below −60°C and desiccating for approximately 27 hours. During the process the temperature of the material undergoing lyophilization should not exceed 35°C.

(3) Seal the vials under an atmosphere of nitrogen. This material represents lyophilized vaccine in final containers.

(4) Store the lyophilized vaccine within the temperature range 1–5°C.

Reconstitution of Lyophilized Vaccine

The lyophilized vaccine is reconstituted for use by dissolving in sterile distilled water.[1] This is supplied with the vaccine in 1.1-ml amounts contained in ampoules of neutral glass with high resistance to thermal and mechanical shock. Constitute the vaccine by withdrawing the entire volume of solvent from an ampoule with a syringe, injecting it into a single final container of lyophilized vaccine, and agitating gently until the dried material is dissolved. The resulting solution represents one human dose of vaccine. The vaccine should be constituted immediately before use.

Expiry Date

The lyophilized vaccine may be used up to 18 months from the date of release from storage. It should be kept between 2°C and 8°C after release.

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[1] This should conform to the specifications for "Water for injection" given in *Specifica-*

The average yield of duck-embryo rabies vaccine prepared according to the foregoing procedure is approximately 18 doses per harvested embryo. However, up to 20% of raw virus suspensions must be discarded because of inherent bacterial contamination of a proportion of eggs. The average vaccine yield is accordingly about 22 doses per sterile harvested embryo.

Potency Test

See chapters 31, 32 and 33.

Annex

PREPARATION, TESTING, AND STORAGE OF MATERIALS AND REAGENTS

Duck Eggs

The duck-rearing industry is much less extensive than the chicken industry and the supply of duck eggs may present difficulties. However, there are good commercial sources that can supply eggs with a fertility rate of approximately 90% during most of the year, although this figure may fall somewhat during the winter months. Duck eggs are in general more sensitive to temperature and mechanical shock than hen eggs and they also withstand storage less well. Since it will usually be necessary to obtain eggs from a distance, attention should be paid to the above points. Non-incubated eggs may be kept at cool room temperature (10–15°C) for up to about 5 days after they have been laid. Viability falls off markedly thereafter and also at temperatures above 15°C.

At the Swiss Serum and Vaccine Institute, eggs from colony-mated Pekin ducks are preferred, although vaccine of satisfactory potency has also been prepared using eggs from similarly bred hybrid birds. It is probable that the eggs of most common breeds of duck can be used, but it is essential that the breed selected develops white feathers since this will determine the appearance of the final vaccine. It is important to use eggs derived from a healthy flock known to be free from *Salmonella* infection. Only naturally clean eggs should be used; they may, however, be treated with an appropriate disinfectant.

Seed Virus

The seed virus is prepared in essentially the same way as the raw virus suspension from which the vaccine is made. Embryonated duck eggs are prepared, inoculated, and incubated, and the embryos are harvested and homogenized as described under *Inoculation and Harvest of Embryos*, p. 245.

Both primary and secondary seed virus consist of a 33% w/v suspension of duck-embryo tissue infected with PM virus at the appropriate passage level. The suspension is prepared in a stabilizing medium (see below).

Seed virus lots are best prepared in volumes sufficiently large to provide a working supply for about 12 months. This procedure is especially recommended for secondary seed lots in order to achieve consistency during actual vaccine production. All seed virus lots are distributed in aliquots of convenient size and frozen and stored at a temperature lower than −70°C.

Samples are removed at appropriate stages during the preparation of each seed virus lot for control testing.

Control tests on seed virus

The tests listed below are performed on each lot of primary and secondary seed virus. Since they are carried out according to standard procedures, full technical details are not given here.

Assay of virus infectivity

This is done on the seed virus after freezing. It is performed in adult mice, which are inoculated intracerebrally. The titre of both primary and secondary seed virus lots should be within the range $10^{6.0}$–$10^{6.5}$ LD_{50} per 0.03 ml. However, preparations with a titre of $10^{5.5}$–$10^{6.5}$ LD_{50} per 0.03 ml may be used to prepare vaccine of satisfactory potency.

Tests for sterility

These tests are performed on the seed virus before freezing. The seed virus is not used for vaccine production until it has been shown to be bacteriologically and mycologically sterile.

Stabilizing Medium

The stabilizing medium is prepared as follows:

Solution 1

dipotassium hydrogen phosphate (K_2HPO_4)	18.75 g
cysteine hydrochloride	7.50 g
lactose	375.00 g
distilled water	2500 ml

(1) Dissolve the potassium phosphate, cysteine hydrochloride and lactose in water. Add each constituent separately in the order listed and

(3) Store in completely filled, tightly stoppered bottles at approximately 4°C.

Solution 2

gelatin	15.00 g
distilled water	2500 ml

(1) Dissolve the gelatin in water warmed to 43–45°C.

(2) Sterilize the warm solution by positive pressure filtration, using a filter and pressure vessel pre-warmed to 36–37°C. Employ a filtration pressure of about 20 N/cm^2 (2 kgf/cm^2).

(3) Store at approximately 4°C.

Preparation of working stabilizing medium

(1) Melt the gelatin (Solution 2) in a water bath maintained at 36–37°C. Cool to approximately room temperature.

(2) Mix equal volumes of Solutions 1 and 2.

(3) Measure the pH of the mixture and adjust it to 7.6 by the addition of 1.0 N sodium hydroxide solution ; approximately 30 ml are required.

(4) Store in completely filled, tightly stoppered bottles at approximately 4°C.

Seed Virus Diluent

The diluent is prepared as follows :

Solution 1

streptomycin sulfate	25.0 g (195×10^5 IU)
distilled water	50.0 ml

Dissolve the streptomycin in water. This will yield approximately 69.5 ml of solution, containing 28 080 IU of streptomycin per ml.

Solution 2

chloramphenicol succinate	6.0 g
distilled water	60.0 ml

Dissolve the chloramphenicol in water. This will yield approximately 64.2 ml of solution, containing 94 000 μg of chloramphenicol per ml.

Preparation of working seed virus diluent, pH 7.2

Hanks' saline without bicarbonate	850.0 ml
sodium bicarbonate solution (4.4%)	48.0 ml
Solution 1	56.0 ml
Solution 2	53.6 ml

(1) Add the reagents to each other in the order listed, mixing well after each addition.

(2) Measure the pH.

(3) Sterilize by positive pressure filtration.

(4) Store in tightly stoppered bottles at approximately −20°C.

Thiomersal

The reagent is prepared as a 1% stock solution :

thiomersal[1]	5 g
distilled water	500 ml

(1) Dissolve the thiomersal in water.

(2) Sterilize the solution by positive pressure filtration.

(3) Store in the dark at approximately 4°C.

VACCINE FOR MAN PREPARED IN HUMAN DIPLOID CELLS

H. KOPROWSKI[1]

In a few countries, inactivated rabies vaccines for man produced in cultures of animal cells are now available for commercial distribution. These vaccines appear, however, to be no more potent than the rabies vaccines produced either in animal nervous tissue or in duck embryo.

One of the principal aims of tissue culture vaccines should be to provide a safer and much more immunogenic product than the rabies vaccines at present available, in order to obtain increased protection of man with a greatly reduced immunization schedule. Recent advances in virological techniques have led to the development of a purified and concentrated vaccine that has these characteristics and is prepared in human diploid cell tissue cultures (strain WI-38). This vaccine has protected monkeys in repeated experiments when administered in a single injection before challenge with street virus.[2] The following description is limited to the production of experimental batches of rabies vaccine prepared in human diploid cell strain (HDCS) WI-38. It should be realized that the general use of such vaccine in man must still await the completion of clinical trials.

Cell Cultures

HDCS WI-38 is propagated and controlled according to the regulations issued in several countries by the authorities responsible for control of biological products.

Seed Lot of Virus

For the production of the seed lot, the Pitman-Moore (PM) strain of rabies virus derived from the virus employed in the production of Semple-type vaccine (see chapter 19) is used, after adaptation to growth in WI-38 cell culture (see chapter 9). The infectivity titre of the virus in the seed lot should not be lower than $10^{6.7}$ LD_{50} per ml. The seed lot should be tested for the absence of adventitious agents according to regulations issued by government authorities for other types of vaccines produced in HDCS.

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[1] Director, The Wistar Institute, Philadelphia, Pa., USA.

[2] See Sikes, R. K., Cleary, W. F., Koprowski, H., Wiktor, T. J. & Kaplan, M. M. (1971) *Bull. Wld Hlth Org.*, **45**, 1.

Infection of Cultures and Propagation of Virus

If the rabies virus in the seed lot is sufficiently concentrated to permit infection of cultures at an input multiplicity of 10 LD_{50} per cell, cultures can be infected as monolayers. At a lower input multiplicity cells have to be infected in suspension.

Infection of monolayers

(1) Remove the culture medium.

(2) Add seed virus at an input multiplicity of 10 LD_{50} per cell in the presence of 100 μg of diethylaminoethyl-dextran (DEAE-dextran) per ml.

(3) Adsorb the virus for one hour at 35–37°C.

(4) Remove the seed virus.

(5) Wash the cells twice with phosphate-buffered saline (PBS).

(6) Add virus propagation medium, consisting of the standard Eagle's Basal Medium[1] containing a double amount of sodium bicarbonate and supplemented with 0.2% of human serum albumin.

(7) Incubate the cultures at 32–33°C for 4 days.

(8) Harvest the infectious fluid.

Infection of cells in suspension

(1) Infect freshly trypsinized cells in suspension with virus at an input multiplicity preferably not lower than 1 LD_{50} per cell in the presence of 100 μg of DEAE-dextran per ml.

(2) Seed the cells in culture flasks and add culture medium (see chapter 9).

(3) Incubate at 36–37°C for 3–4 days until a complete monolayer is formed.

(4) Trypsinize the cells and replant in equal portions in two new culture flasks.

(5) Incubate for 3 days at 36–37°C in culture medium (see chapter 9).

(6) After washing the cultures twice with PBS, replace the culture medium by virus propagation medium.

(7) Incubate the cultures for an additional 3 days at 32–33°C.

(8) Harvest the infectious fluid.

The harvested virus is freed from cells and cell debris by low-speed centrifugation (see chapter 15).

Concentration and purification of the virus

The virus can be concentrated and purified by any of the three methods described in detail in chapter 15 :

(1) precipitation by zinc acetate ;
(2) sedimentation by high-speed centrifugation ;
(3) concentration by ultrafiltration through a nitrocellulose membrane.

It will not be possible to determine the exact concentration factor until clinical trials have been completed. Infectious culture fluids have been concentrated up to 400 times, with corresponding recovery of protective activity of the final vaccine.

Inactivation of the virus

At The Wistar Institute a fresh 1 : 40 solution of β-propiolactone in cold sterile distilled water is immediately added to the refrigerated virus suspension to give a final concentration of 1 : 4000. The mixture is constantly stirred in the cold by a magnetic stirring bar placed in the virus container.

The treatment is continued for 18 hours at 4°C, and is followed by incubation of the inactivated virus suspension at 37°C for 2 hours in order to hydrolyse the β-propiolactone completely.

In principle the virus can be inactivated either before or after concentration and purification. If the ultrafiltration method is employed for virus concentration and purification, however, and the virus is inactivated before ultrafiltration, care has to be exercised to avoid formation of aggregates.

The virus can also be inactivated by ultraviolet irradiation (see chapter 25), by gamma irradiation, or by other chemical inactivators, provided that these have been approved for use in vaccines produced for man.[1]

Safety and potency tests

There are no regulations as yet concerning standard safety and potency tests for human vaccines produced in HDCS. The reader is referred to the discussion of such tests in chapter 30.

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[1] Wiktor, T. J., Aaslestad, H. G. & Kaplan, M. M. (1972) *J. appl. Microbiol.*, 23, 914.

Annex

PROCEDURE FOR PREPARATION OF 1000 ml OF RABIES VIRUS SUSPENSION IN CULTURES OF WI-38 CELLS

Materials

Seed virus : PM strain of rabies adapted to HDCS, with an infectivity titre of not less than $10^{6.7}$ LD_{50} per ml.

Cells : WI-38 cultures propagated in Blake bottles.

Culture medium : Eagle's Minimum Essential Medium [1] in Earle's salt solution [2] supplemented with 10% calf serum and 5×10^7 IU of chlortetracycline per litre.

Virus propagation medium : Eagle's Basal Medium [2] in Earle's salt solution [2] containing a double amount of sodium bicarbonate and supplemented with 0.2% human serum albumin and 5×10^7 IU of chlortetracycline per litre.

Trypsin : 0.25% solution of trypsin in Basal Medium.

Procedure

Virus propagation

Trypsinize cells from 5 l-litre Blake bottles and suspend them in 50 ml of culture medium. A cell concentration of approximately 1×10^6 cells per ml will be obtained. Add enough seed virus with DEAE-dextran (see p. 257) to obtain an input multiplicity of 1 LD_{50} per cell.

Mix and allow adsorption to proceed for 30 minutes at 36°C.

Mix and distribute 5×10^6 cells into 10 l-litre Blake bottles. Add 100 ml of culture medium per bottle and incubate at 36°C for 3 days. Remove the culture medium, trypsinize the cells and replant into 20 1-litre Blake bottles ; feed cell propagation medium and incubate for 3 days at 36°C.

Remove the medium. This rabies-infected fluid can be used as a seed virus for the next lot of vaccine.

Wash cells twice with PBS solution.

Add virus propagation medium (50 ml per bottle) and incubate at 32–33°C for 3 days.

Harvest the virus.

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[1] See chapter 9, p. 110.

Centrifuge the infected tissue culture fluid for 30 minutes at 1000 *g* in a refrigerated centrifuge, or filter through a Millipore SM membrane of 5.0 µm porosity.

Virus inactivation

Refrigerate the virus suspension at 4°C.

Mix 1 ml of β-propiolactone with 40 ml of cold sterile distilled water and add 10 ml of this solution to 1000 ml of virus. Mix well and keep at 4°C for 18 hours.

Transfer the inactivated virus to a 37°C water bath and keep at this temperature for 2 hours.

Virus concentration

See above and chapter 15.

CHAPTER 29

TISSUE CULTURE VACCINES FOR ANIMALS

M. K. ABELSETH [1]

The advent of tissue culture techniques has brought an upsurge of activity in rabies virus research. Attempts to grow rabies virus in cell cultures were made early in the present century, but with little success (Levaditi, 1913), and the first significant advance was made by Kissling (1958), who was able to obtain mouse titres of $10^{-3.5}$ LD_{50} utilizing the CVS strain in primary cultures of hamster kidney. Subsequently, a number of investigators reported the growth of fixed and field strains of rabies virus both in primary and in continuous cell cultures from a variety of tissues. These accomplishments resulted in the development of several types of rabies vaccine for the immunization of animals.

Since little destruction of cells by rabies virus occurs during multiplication, most vaccines produced in this manner are relatively free from extraneous proteins, a factor that may be of importance if repeated vaccinations are required.

Several types of rabies vaccine for animals are produced in tissue culture :

Cell system	*Virus strain*	*Type*	*Recommended for*
chick fibroblast	LEP Flury	live	dogs
dog kidney (cell line)	HEP Flury	live	dogs, cats, cattle
porcine kidney	ERA	live	dogs, cats, cattle, sheep, horses, goats
hamster kidney	LEP Flury	live	dogs
hamster kidney	SAD [a]	live	dogs, cattle
hamster kidney	CVS	inactivated	cats, dogs, sheep, goats, cattle, horses

[a] This vaccine, produced in the USSR, is undergoing field trials.

Experimental evidence suggests that the virus propagated in tissue culture is more antigenic than that produced by other methods (Abelseth, 1967 ; Cabasso et al., 1965 ; Dean et al., 1964).

Although long-term immunity trials have not been carried out with all types of tissue culture vaccine, evidence for some types indicates a duration of immunity at least equal to that of LEP Flury vaccine produced in chick embryo.

As with all vaccines, there are certain requirements that must be met in order to obtain a safe and effective product :

(1) clean, properly stored seed strains in which the passage level has been established ;

(2) production cell cultures free of contaminating organisms ;

(3) production protocols as developed in experimental tests ;

(4) adequate testing ;

(5) proper storage and handling.

Vaccine Production

The procedure for vaccine production varies with the type of cell culture and virus strain utilized. The first criterion is familiarity with the production of large-scale cell cultures, including knowledge of the variation in media, pH, and time and temperature of incubation. After the cells have been seeded with virus, further variations may be necessary to facilitate the maximum yield of virus. The ideal time for harvest and proper freeze-drying techniques must also be determined.

As a model for the production of modified rabies vaccine in tissue culture, the protocol developed for the ERA strain (Abelseth, 1964) in porcine kidney is described. As indicated previously, this procedure may need to be varied depending upon the cell culture and virus strain utilized.

Source and Preparation of Cells

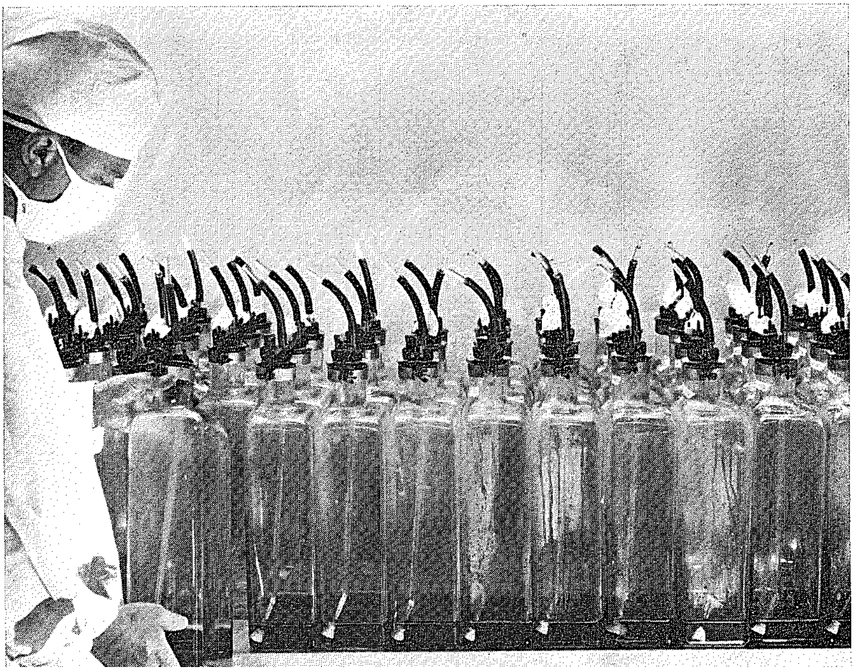
Select a healthy pig, 1–3 months of age. Anaesthetize and exsanguinate. Remove both kidneys aseptically and transfer to a sterile room or hood. After stripping off the capsule, harvest the cortex (sterile, sharp, curved scissors are convenient for this purpose) and mince with a second pair of sharp scissors to 1 mm^3 or less.

Place the minced kidney tissue in a 250–500-ml conical flask containing 100–200 ml of 0.25% trypsin at 32–34°C. This temperature should be maintained throughout the trypsinizing process. Stir for 20–25 minutes with a magnetic stirrer. After the tissues have settled, discard the first trypsinization solution, thus removing most of the blood and fragmented cells.

Carry out further trypsinizations, with 100–250 ml of 0.25% trypsin for each. For each operation, decant the trypsin solution containing the cells after 15–25 minutes of trypsinization and filter through sterile cheese cloth to hold back cell clumps. Centrifuge the solution for approximately 3 minutes at 60 *g*. Higher speed and longer centrifugation will cause the cells to pack too firmly for dispersion. Discard the trypsin and wash the packed cells in phosphate-buffered saline (see Annex, page 266). Centrifuge again and decant the saline. Repeat the harvesting process until the required amount of cells is obtained. As cells are harvested, they should be kept cool until processing has been completed. One pair of kidneys normally yields 8–10 ml of packed cells. When sufficient cells have been obtained, add 1.25 ml of packed cells per 1000 ml of growth medium, which consists of Hanks' balanced salt solution with lactalbumin hydrolysate (see Annex), 5% of normal bovine serum, 1% of a 2.8% solution of sodium bicarbonate, benzylpenicillin potassium (200 IU/ml), and nystatin (25 IU/ml). The final pH of the growth medium should be 7.2–7.4. The mixture of cells and medium should be prepared in a single container to ensure uniformity of cell suspension.

Dispense the suspended cells into sterile Blake or Povitsky bottles (Fig. 1) at the rate of 100 or 400 ml, respectively, per bottle. Other types of bottle may be used with comparable amounts of cell suspensions. Maintain the bottles in a stationary position for 5–6 days at 37°C, at which time the cell sheets should have become confluent. Check the confluency and appearance of the cells (Fig. 2) by examining all the bottles using an inverted

FIG. 1. POVITSKY BOTTLES USED IN VACCINE PRODUCTION



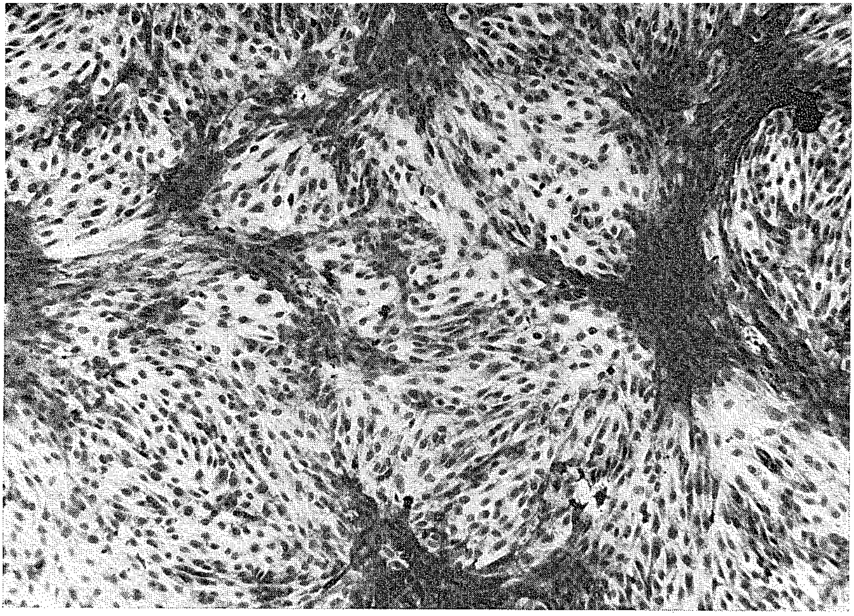
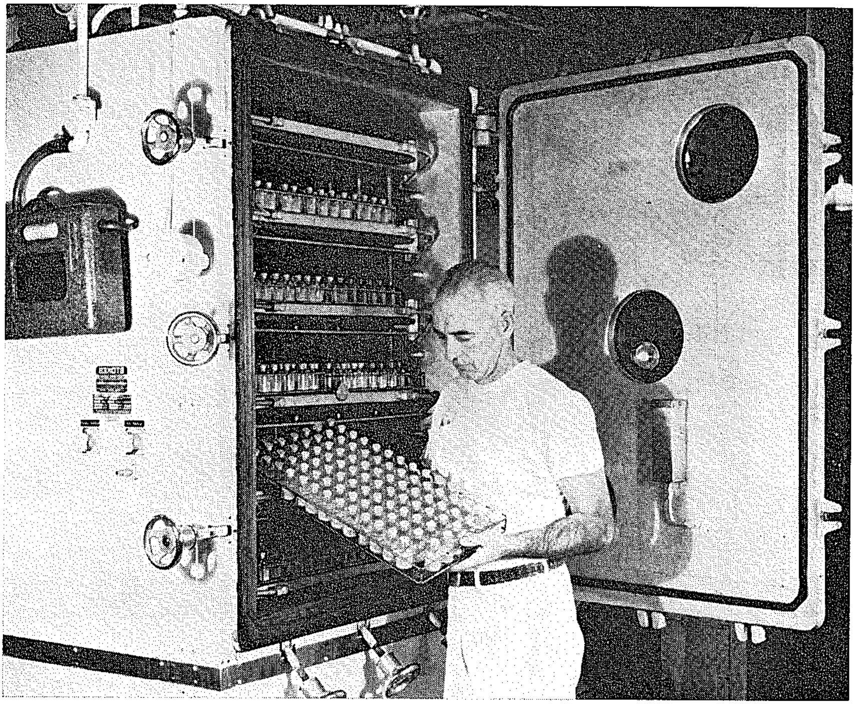


FIG. 3. VACCINE BEING REMOVED FROM THE FREEZE-DRYING CABINET



microscope. Any bottles in which the cells have not become confluent or do not appear healthy should be discarded. Such findings may be attributed to improperly washed bottles or perhaps to poorly fitting stoppers. If a large number of bottles with poor cells are found, discard the entire batch. It is not advisable to extend the incubation period in order to improve the cells since complete cell sheets may overgrow and become granular, resulting in inadequate virus production, or they may fall off the glass before production is complete.

Infection and Incubation of Pig Kidney Cells

The medium in the Blake bottles is replaced by 100 ml of medium identical to that used for growth except that it contains 2% of normal bovine serum and 5% of sodium bicarbonate. The medium in the Povitsky bottles is replaced by 500 ml of medium containing 2% of bovine serum and 3.5–4% of sodium bicarbonate.

Infect the cells by adding seed virus direct to the media in the bottles. The amount of seed will depend on the titre of the seed stock. Incubate the cells at 37°C for 6 days. Change the maintenance medium on the 3rd day and discard. On the 6th day following infection, harvest the fluids and add fresh medium to the cells. Repeat after a further incubation of 4 days. This process may be repeated again, depending on the condition of the cell sheet. More than one harvest may be obtained from a cell preparation, since the release of rabies virus into the fluid causes little cell damage. Such techniques require constant control of growth conditions. Each harvest should be considered as a separate lot of vaccine and tested separately.

Final Preparation of Vaccine

The harvest is dispensed into single-dose (2.0-ml) or multiple-dose (14.0-ml) vials by means of a Brewer automatic pipetting machine. The filled vials (in trays) are placed in an alcohol bath cooled to approximately −50°C as indicated by a dial thermometer placed in one of the vials. The frozen vaccine is transferred to the freeze-dryer (Fig. 3) and desiccated under high vacuum and at a temperature not exceeding 30°C in the final stages of drying. Careful control of temperature and pressure is maintained throughout the drying cycle to obtain the maximum drying rate. The drying cycle requires 24–48 hours. The vials are closed with sterile rubber stoppers under vacuum and then capped with a single aluminium seal. The finished vials are checked for imperfections and packed in boxes labelled for identification of product, serial number, quantity, and filling

Thirty vials are submitted for quality control testing; the remainder of the lot is stored at 4°C pending completion of the tests required for release. Once released, the unlabelled vials are suitably identified and packaged. The label on each vial should indicate:

the name of the vaccine;
the animals for which it is recommended;
the minimum age of animal to be vaccinated;
dosage;
route of administration;
expiry date;
lot number.

A leaflet should be inserted into each package with any further recommendations regarding proper handling, reconstitution, and revaccination.

Diluent

Since this is a lyophilized product, a diluent must be supplied in each package. The diluent consists of pyrogen-free distilled water dispensed into vials, which are closed with rubber stoppers and sealed with an aluminium seal. The filled, stoppered, and capped vials are sterilized by autoclaving.

Annex

PREPARATION OF REAGENTS

Phosphate-buffered saline

sodium chloride	8.0 g
potassium chloride	0.2 g
sodium hydrogen phosphate (Na_2HPO_4)	1.15 g
potassium dihydrogen phosphate (KH_2PO_4)	0.2 g
calcium chloride	0.1 g
magnesium chloride hexahydrate	0.1 g
streptomycin sulfate	0.2 mg (15.6 IU) per ml
neomycin sulfate	0.008 mg (5.36 IU) per ml
distilled water to make	1000 ml

The solution is sterilized by Seitz filtration through Carlson-Ford High Purity filter sheets, grade HP/EKS. It has a pH of 7.5±0.1.

Hanks' balanced salt solution with lactalbumin hydrolysate

sodium chloride	8.0 g
potassium chloride	0.4 g
calcium chloride	0.14 g
magnesium sulfate heptahydrate	0.2 g
potassium dihydrogen phosphate (KH_2PO_4)	0.06 g
sodium hydrogen phosphate (Na_2HPO_4)	0.0475 g
glucose	1.0 g
phenol red	0.02 g
lactalbumin hydrolysate	5.0 g
streptomycin sulfate	0.2 mg (156 IU) per ml
distilled water to make	1000 ml

After filtration, the solution is ready for use.

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PART V

VACCINE SAFETY AND POTENCY TESTS

CHAPTER 30

GENERAL CONSIDERATIONS IN SAFETY AND POTENCY TESTING

K. HABEL[1]

Safety tests on biological products are designed to detect any material or property that may be harmful to the recipient, such as bacterial contamination, infectious virus, or toxicity. In the case of viral vaccines in general, and especially of rabies vaccine, the specific problem of residual virulent virus is of the utmost importance. Besides rabies virus, which is intentionally present in some form, the vaccine might contain other infectious agents that were present in the original animal or tissue culture source. In inactivated virus vaccines the test for residual virulent virus is relatively simple and direct, using the most sensitive procedure for demonstrating rabies virus—the intracerebral inoculation of young mice. Fermi-type vaccine represents a special case : although it is made from a virus strain known to be virulent for man if given in a sufficiently large dose, it is not completely inactivated during preparation and a small amount of live virus is purposely allowed to remain in the final vaccine. It is extremely important, however, that the amount of infectious rabies virus should not exceed the specified level. In the case of live attenuated virus vaccines, the problem is rather more complex because a minimum amount of infectious virus is required; it must be demonstrated, however, that this virus is avirulent for the intended recipient. Vaccines for veterinary use can be tested by direct inoculation of the species in which they are to be used.

Procedures giving absolute assurance that a vaccine for use in man is free of all possible extraneous infectious viruses can be very complex and costly. Such procedures can, however, be limited to what is reasonable and practical by taking advantage of the extensive field experience with a particular tissue or animal source. Rabies vaccines from a tissue culture source must pass the same safety requirements as other tissue culture vaccines intended for human use.

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[1] [illegible]

and propose guidelines. Specific minimum requirements and regulations should be laid down by individual national authorities.

Although the need for evaluating the immunizing potencies of rabies vaccines has been recognized since the early Pasteur days, and practical standardized tests have been available and in use for over 25 years, many laboratories producing vaccines still do not make a routine practice of potency testing their products. Merely following a described standard vaccine production prodecure does not necessarily ensure that the product will consistently have satisfactory potency levels. Many laboratories that have been producing vaccines for years have been amazed on first testing their products to find that they had low or negligible potencies.

It has always been difficult to evaluate vaccines on the basis of results obtained in human beings under field conditions because of (*a*) the lack of true controls, (*b*) the relatively small number of human cases of rabies in a given area, and (*c*) the impossibility of accounting for all the factors that impair the comparability of human exposures. Furthermore, the need is really for an evaluation of a vaccine *before* it is released for human or veterinary use.

There would appear to be 3 important considerations in assessing any potency test of rabies vaccines. First, the test procedure should actually evaluate the property of the vaccine that determines its effectiveness in the prophylaxis of rabies in man or animals, and preferably in a quantitative way. Using a naturally susceptible host, the ideal test would simulate conditions of natural exposure and usual prophylactic treatment. This would mean the use of street virus introduced through a bite wound, followed by daily doses of those vaccines intended for human use. This has, of course, been found to be impracticable, as have most types of test where administration of vaccine is started *after* experimental exposure of the test animal. Most tests, therefore, involve multiple doses of vaccine (as administered in man), followed by subsequent challenge with fixed virus given intracerebrally — a more easily standardized type of challenge. While far from reproducing the conditions of natural exposure and the standard schedule of vaccine administration, this type of test does reflect fairly closely the ability of a vaccine to protect under natural conditions. On the other hand, the chicken-embryo live virus vaccines, as used in veterinary practice, are administered prior to exposure in a single dose and depend upon multiplication of the attenuated virus for their immunizing effect. Therefore, potency tests of these vaccines require the use of animals in which the vaccine virus strains are avirulent yet capable of multiplying. The test involves a single dose of vaccine followed by a subsequent challenge with virus introduced by the peripheral route.

The second important factor in a potency test is its practicability— the ease with which it is carried out, the availability of the material used, and the cost and time involved. Not all laboratories can obtain large numbers of experimental animals easily, nor can they, perhaps, support the expense of repeated tests when animal costs are high. The time factor is important, since newly prepared vaccine must be held until potency tests are complete and the time required for this has to be deducted from its period of effectiveness.

The third requirement is for standardization of the test procedure so that the results obtained with different vaccines will be comparable, both within a single laboratory and between different laboratories.

Through experience it has been found that the two most important variables that can markedly influence the results of any type of rabies potency test are the animals and the challenge virus employed. For optimum results, the animals must be in good general health and of the correct age. Uniformity of response among the individual animals making up the test group does not necessarily require highly inbred strains, but all animals should be from the same closed colony stock. Another strain factor that has proved troublesome in the mouse tests is the variation from colony to colony in the ability of the mice to respond with a high degree of immunity to a given vaccine. Obviously, for test purposes a strain of mice should be used that has been shown to respond efficiently to a known potent vaccine.

Variation in the degree of demonstrable immunity has also been shown to depend upon the strain of virus used to challenge the immunized animals. This variable has been eliminated by the development of a standard challenge virus—the CVS fixed virus derived from the original Pasteur strain.[1] The proper technique for preparing and handling the standard challenge virus is given in chapter 33.

In the following chapters of Part V several different potency tests are described. The first 3 are mouse tests primarily used for testing inactivated virus vaccines intended for human use. They are also satisfactory for potency tests of inactivated vaccines for veterinary use. The decision as to which of these 3 tests to use in any particular laboratory will depend on the type of information required and the facilities available. If a laboratory has limited facilities for animal procurement, and wishes merely to screen its vaccines for potency or lack of potency, then the non-quantitative modified Habel test should be used (chapter 32). If facilities are available and a quantitative evaluation of potency is desired as a continuing check on efficiency of vaccine production, then one of the more standardized tests is indicated. The original Habel test (chapter 31) and the NIH test (chapter 33) are both quantitative; the former is of the immunity-breakdown type where all animals receive the same immunization and the challenge where varying amounts of vaccine are used and all mice receive the same challenge. Both these quantitative tests require larger numbers of mice and should include a standard vaccine run in parallel with the unknown one under test. The inclusion of a standard vaccine control is absolutely essential in the NIH test. Under ideal conditions, the antigen-extinction NIH test is the most standardized of existing tests and gives results of high comparability.

The test described for use with modified live-virus vaccines (chapter 34) also represents a screening, non-quantitative test in guinea-pigs. Another important part of the potency testing of all live attenuated virus vaccines is the titration of the quantity of infectious virus to ensure that it reaches a required minimum.

Should a laboratory wish to test its vaccine in the species in which it will eventually be used in practice, this can be done directly with veterinary vaccines. Immunization of the species can be done as in the field, followed by intramuscular challenge with street virus, including an adequate number of vaccinated and control animals to give statistically significant results. However, this is a difficult procedure that should be carried out only by a laboratory actively engaged in rabies research. The nearest approach to such a direct test that can be made in man is to immunize individuals according to the recommended schedule and then bleed them at intervals to determine the rapidity and degree of neutralizing antibody response.

Special problems may arise in the use of tests for new vaccines developed from tissue culture sources and for vaccines made with purified virus. Where a comparison is being made, as in the NIH test, between the production batch and a standard vaccine, the dilutions of vaccine used and the degree of antigenicity found cannot be expressed in milligrams of tissue. Therefore, a "dilution factor " will have to be applied so that the production vaccine and the standard are compared in relation to the dose that would be used in practice. Furthermore, since the standard vaccine is currently of the brain tissue type, it may eventually be necessary to introduce a second standard of tissue culture or purified virus origin for the potency testing of new vaccines. The crude brain emulsion may have an inhibitory or even adjuvant effect on the antigenicity of the standard vaccine, but this would not occur with more highly purified test material.

At present, tissue culture vaccines for man are subjected to the Habel or NIH potency test. As already indicated, it is desirable to develop an international reference preparation produced in tissue culture and tested for antibody production in man. Batches of vaccine could then be tested as suggested above. Ultimately the newly-developed antibody binding test (chapter 35) will probably also be employed for evaluating the potency of tissue culture vaccines.

As regards safety requirements for newly-developed tissue culture vaccines for use in man,[1] the standard tests required by national authorities for well-established inactivated viral vaccines produced in tissue culture, such as poliomyelitis, measles, and rubella vaccines should be employed for rabies vaccines as well. These procedures, to be carried out after inactivation, should include tests for: bacterial sterility; absence of mycoplasma; absence of living rabies virus and adventitious viruses; concentration of adjuvant, if employed; level of heterologous animal serum, if employed for viral growth; and any extraneous substances, e.g., antibiotics, protein.

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[1] See, for example:

Bergsma, D., ed. (1966) *Chicago Conference: Standardization in Human Cytogenetics, Chicago, 1966*, New York, National Foundation—March of Dimes (*Birth Defects: Original Article Series*, Vol. II, No. 2);

International Association of Microbiological Societies, Permanent Section on Microbiological Standardization, Committee on Cell Cultures (1969) *Minutes of the Sixth Meeting, New York*;

United Kingdom (1971) *Therapeutic substances: the Therapeutic Substances (Manufacture*

HABEL TEST FOR POTENCY

K. HABEL[1]

Mice

White Swiss mice, 4–6 weeks of age, and uniform in weight, are used; they may be selected at random, irrespective of sex, or mice of only one sex may be used if preferred.

Immunization Procedure

Sixty mice receive 0.25 ml of a vaccine diluted to give a 0.5% emulsion of original wet-weight of brain. Inoculations are given intraperitoneally on Monday, Wednesday, and Friday during 2 successive weeks (a total of 6 doses). Thirty mice should be kept apart from the rest at the beginning of the immunization for use as controls at the time of challenge. When testing vaccines prepared from material other than brain tissue, the vaccine should be diluted 1 : 10 before inoculation of 0.25 ml per dose.

Challenge

A challenge test is performed on the 14th day from the first dose of vaccine. At this time 2 ampoules of the standard challenge fixed virus (see chapter 33) should be thawed out and diluted to a 10^{-1} suspension. Serial tenfold dilutions from 10^{-1} to 10^{-7} are then made, using 2% heat-inactivated horse or rabbit serum in distilled water as diluent. It is recommended that the dilutions of challenge virus be held in an ice-water bath during the performance of the test to prevent loss of virus titre. It is important to use a separate pipette for transferring and mixing the material at each step in the serial dilution series.

Groups of 10 vaccinated mice are then challenged intracerebrally with 0.03 ml of the 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} dilutions of virus in that order. A single syringe and needle may be used for all inoculations, provided the empty syringe is rinsed several times in the next dilution before being refilled.

With a new syringe the control mice are then inoculated with the 10^{-7}, 10^{-6}, and 10^{-5} dilutions of virus in order to determine what dilution represents the LD_{50}. If the challenge virus is fully active, these 3 dilutions will usually give survival rates ranging from 100% to less than 50%. Scattered deaths at all 3 dilutions should be viewed with suspicion as indicating errors in the dilution of virus. For the potency test to be valid, the 50% endpoint dilution (corresponding to 50% rabies mortality) should be beyond the 10^{-5} dilution in the control mice.

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[1] *Formerly* Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif., USA.

All mice are observed for 14 days, and only deaths occurring after the fifth day should be considered as rabies deaths. Mice should be observed daily and symptoms recorded. When making potency calculations, mice surviving at the end of 14 days but showing definite symptoms of rabies should be considered as having died of rabies.

Determination of Degree of Protection

50% endpoint dilutions in vaccinated and in control mice are determined by the methods described in the Appendix 1, p. 321. These methods give the dilutions at which, theoretically, 50% of the mice would die of rabies, the calculations being made on the basis of the actual results in the test with the dilutions employed. By subtracting the log of the 50% endpoint in the vaccinated animals from that in the controls, the log of the "LD_{50} of protection" is readily obtained. To meet minimum standards, the difference should be 3, or log 1000, indicating that the vaccine affords protection against 1000 LD_{50}.

Modification of Technique for Live-Virus or Attenuated Vaccines

Where the vaccination schedule prescribes gradually increasing doses, as in the original Pasteur type of vaccine, the 6 intraperitoneal doses of 0.25 ml of equivalent 0.5% emulsion should be made starting with the least virulent of the vaccine doses and grading to the most virulent. If all doses in the vaccination schedule are the same, no change from the original procedure is necessary.

MODIFIED HABEL TEST FOR POTENCY

K. HABEL[1]

For many laboratories producing antirabies vaccine, the need for relatively large numbers of experimental animals makes the routine use of the standard mouse test for potency impractical. Especially is this true in those laboratories where multiple small volumes of vaccine are prepared at frequent intervals. In these situations it would be of value to have available a simplified potency test that would screen out vaccines of below-standard antigenic potencies and would at the same time be comparable with the more complete standard test in respect of minimum requirements.

In checking the results of many potency tests with vaccines of high, low, and intermediate levels of antigenicity, it has been apparent that approximately 500 LD_{50} as an intracerebral challenge dose kills less than half the immunized mice if the potency of the vaccine determined by the complete test is over 1000 LD_{50} of protection, which is the minimum requirement.

The technique of the test is the same as the standard procedure (see chapter 31) using 6 intraperitoneal doses of 0.5% suspension of nervous tissue (0.25 ml), and intracerebral challenge on the 14th day followed by 14 days of observation. Only 20 mice are immunized, and 15 additional mice are held as controls. On the 14th day the standard challenge virus (see chapter 33), diluted to contain 500 LD_{50} per 0.03 ml, based on past experience with a frozen virus pool, is given intracerebrally to the vaccinated mice, and at the same time groups of 5 control mice receive the challenge virus diluted to 10^{-5}, 10^{-6}, and 10^{-7} (see chapter 31 for an analogous procedure).

For the test to be valid, the control titre should indicate that the challenge dose given to the vaccinated mice was between 100 and 1000 LD_{50}; and 50% of the vaccinated mice should survive in order that the vaccine pass the screening potency test (for calculation of challenge dose, see chapter 40).

It is recommended that at periodic intervals a complete standard potency test (see chapters 31 and 33) be performed to obtain a more quantitative evaluation of the vaccine over long periods of production in each laboratory.

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CHAPTER 33

THE NIH TEST FOR POTENCY*

E. B. SELIGMANN, Jr[1]

The Standard Challenge Virus

The standard challenge virus (CVS) is supplied periodically, usually once a year, to laboratories in the USA.[2] This provides a nearly uniform challenge virus and makes it possible to evaluate different lots of vaccine as well as vaccines from different laboratories. Laboratories are urged to follow closely the procedures outlined.

The Working CVS

CVS is supplied as a 20% mouse-brain suspension in a diluent containing 2% of horse serum in distilled water. This has been stored in solid carbon dioxide (dry ice) before shipment; it should be used only if received in the frozen state. It is also distributed as a freeze-dried preparation when there is the possibility that the shipment will thaw before receipt. The ampoules of the dried preparation contain 0.5 ml of a 20% mouse-brain suspension otherwise identical with the frozen preparation. The contents of the ampoule of frozen virus should be thawed rapidly under cold running water and then diluted 1 : 2 with 2% horse-serum diluent. This yields a 10% suspension. The supernatant fluid is diluted to yield a 10^{-3} dilution of the virus and, using 0.03 ml of this dilution as the inoculating dose, a sufficient number of normal mice are inoculated intracerebrally to produce the amount of working CVS needed for approximately one year. (One mouse brain will yield approximately 1.5 ml of a 20% suspension.) In order to ensure a high titre in the working preparation when starting with freeze-dried seed virus, it is best to make two mouse passages and use the second passage for the working CVS. Mice will show paralysis in 6–10 days. When an inoculated mouse is completely paralysed but still breathing, the brain is placed in a common container and when the collection is complete they are thawed, weighed, and ground to pulp; enough of the 2% horse serum diluent is then added slowly, while grinding, to yield a 20% suspension. The suspension is given a lot number and without straining or centrifuging it is distributed into ampoules. The ampoules are flame-sealed, quick-frozen, and stored at dry-ice temperature (approximately −70°C).

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* This test was originally developed at the United States National Institutes of Health, Bethesda and much of the text of this chapter has been adapted from : United States National Institutes of Health (1953) *Minimum requirements : rabies vaccine*, 3rd rev., Bethesda, Md., USA.

[1] Director, Division of Control Activities, Bureau of Biologics, Food and Drug Administration, Rockville, Md., USA.

Each step in preparing the working CVS must be carried out promptly so as to ensure the survival of the maximum possible amount of virus. Before use as challenge virus, the LD_{50} value of the lot should be determined in 6-week-old mice. The lot is satisfactory provided that the LD_{50} value is between the $10^{-6.0}$ and $10^{-8.0}$ dilutions, inclusive. The maximum variation from test to test in the titre obtained should not exceed one tenfold dilution when the same lot of challenge virus is used. The working CVS may be stored for as long as full potency is maintained as shown by mouse titration. This is essential in order to ensure uniformity of the working CVS among all laboratories producing rabies vaccine.

Reference Vaccine

An International Reference Preparation of Rabies Vaccine (ultraviolet-light-inactivated and freeze-dried) is available to national laboratories on request from the International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen.[1] When the contents of each ampoule are resuspended in 8 ml of distilled water, the vaccine is considered to be a 10% suspension. The dried vaccine is stored at 2–10°C at all times and after reconstituting to the liquid state should be used immediately. If it is desired to perform the test with a single ampoule of the reference vaccine, a 5% suspension may be prepared, part of which is used to prepare further dilutions for use in the first immunization. The remaining portion of the 5% suspension is frozen and held at −70°C for use in the preparation of dilutions at the time of the second immunization.

Immunization of Mice

The initial suspension of the reference vaccine (10%) has to be adjusted to the same concentration of brain tissue as the vaccine submitted for test, unless the volumetric method is used (see page 285). For inoculation of mice, dilutions of both the test vaccine and the adjusted reference vaccine are prepared in parallel.

Three or more dilutions of each vaccine under test are prepared in phosphate-buffered saline [2] (pH 7.6), using fivefold increments. The range of dilutions is selected so that the middle dilution will contain enough vaccine to protect 50% of the mice. The range will depend upon the strength of the challenge dose, the kind of mice used, and the potency of the vaccine. With a challenge dose in the range 5–50 LD_{50} (see below) a dilution range of 1.0%, 0.2%, and 0.04% of brain tissue has worked well for the reference vaccine and commercial vaccines produced in the USA.

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[1] In the USA a similar Reference Rabies Vaccine is distributed to vaccine producing laboratory by the Division of Control Activities, Bureau of Biologics, Food and Drug Administration, Rockville, Md., USA.

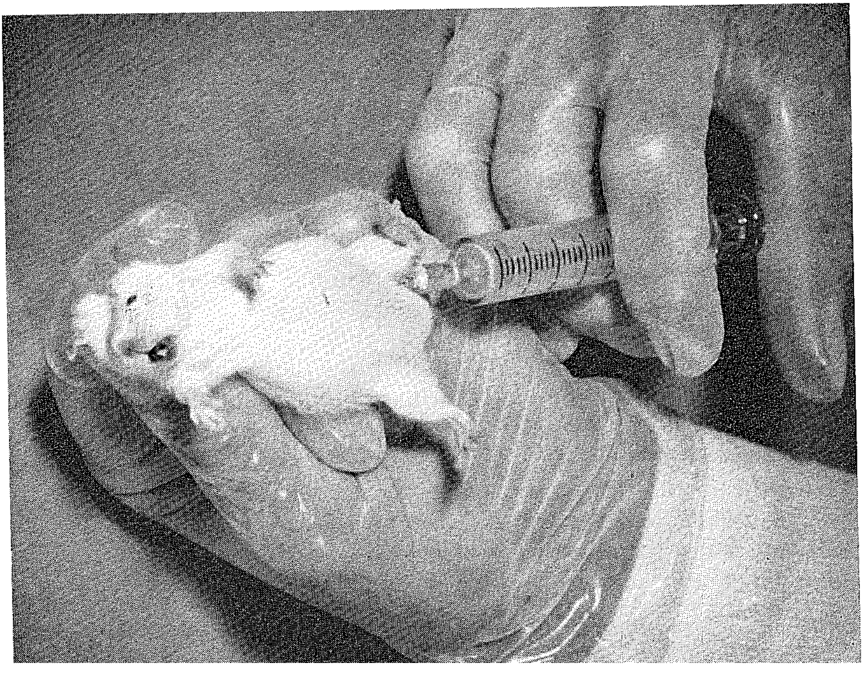
[2] 8.5 g of sodium chloride dissolved in 15 ml of M/15 KH_2PO_4, 85 ml of M/15 $Na_2HPO_4 \cdot 12H_2O$ and 900 ml of distilled water.

At least 16 mice approximately 4 weeks old and weighing 11–15 g are injected intraperitoneally with 0.5 ml of each dilution of vaccine. Two doses of vaccine are given to each mouse one week apart. Enough control mice are set aside at the time the test mice receive the first dose of vaccine for an adequate titration of the challenge virus to be made with at least 10 mice for each dilution of virus (a total of 30–40 control mice).

Mice are vaccinated with 0.5 ml intraperitoneally (see accompanying figure). The needle best suited for vaccination is the 0.50-mm × 16-mm (25-gauge, $^5/_8$-in) needle. The vaccine suspension should be injected rapidly. If there is any doubt as to whether the needle point is in the peritoneal cavity, the point should be moved from side to side and the skin observed.

If a single needle and syringe are used for inoculating the mice with the vaccine, mice receiving the most dilute vaccine should be inoculated first,

INTRAPERITONEAL INOCULATION OF A MOUSE



each mouse is clearly marked, mice receiving each vaccine concentration should be housed separately from mice receiving other concentrations.

Challenge of Control and Test Mice

All mice are challenged intracerebrally 14 days after the first dose of vaccine.

One ampoule of the pooled working CVS is thawed rapidly under cold running water and diluted 1 : 6 with 2% horse serum in distilled water. This mixture is then centrifuged for 5 minutes at a relative centrifugal force of approximately 1500 *g*. The supernatant fluid is used to make further dilutions. All dilutions are made in the same diluent as originally used. It is recommended that the dilutions of the challenge virus be held in an ice-water bath, or its equivalent, during the performance of the test in order to prevent potency loss.

The immunized mice are challenged with a dilution of virus that has been found by routine tests to contain between 5 and 50 LD_{50} of virus.[1] Each laboratory, therefore, must determine for itself the dilution of working CVS that will contain a test dose of between 5 and 50 LD_{50} of virus. The initial 1 : 6 dilution may be varied in accordance with experience. Serial tenfold dilutions of the supernatant are then made and the one found to contain the test dose of virus in 0.03 ml used for challenge. Experience in one laboratory with the working CVS prepared in that laboratory has led to the use of a 1 : 6 initial dilution and use of the 10^{-4} dilution of the supernatant for challenge.

After all immunized mice have been inoculated with the test dose of virus, one group of control mice is inoculated with the same test dose of virus. Three tenfold dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of the test dose are then prepared for inoculation of the remaining control mice. It is desirable to inject first the 10^{-3} dilution, then the 10^{-2} dilution, and lastly the 10^{-1} dilution of the test dose of virus. In this way one syringe may be used to challenge all the immunized mice and one group of control mice ; another syringe must be used to challenge the remaining control mice.

All mice are observed for 14 days from the time of the challenge injection. Only those deaths occurring after the fifth day and those preceded by signs of fixed-virus rabies (paralysis, convulsions) are considered deaths from rabies. However, any mice becoming paralysed but surviving the 14-day observation period are also counted as deaths from rabies.

The definitions of "paralysis" and "convulsions", as applied to mice following the injection of the challenge virus, are as follows :

Paralysis is the partial or complete loss of motor power of one or more legs.

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[1] For calculation of the challenge dose see chapter 40, page 315.

Convulsions are indicated by violent and abnormal muscular contractions of the body—often termed spasms. These are brought about by external stimulation, such as a disturbing sound or handling.

For the test to be considered valid, the results obtained after challenge of the immunized mice must show that the dilutions of the reference vaccine encompass the 50% endpoint—that is, the majority of the mice receiving the highest dose of vaccine survive, and the majority of the mice receiving the smallest dose of vaccine die. For the test vaccine, unless an exact endpoint is especially desired, it is only necessary that the majority of the mice receiving the highest dose of vaccine survive. The virus titre of the test dose of challenge virus is not particularly important, provided that it falls between 5 and 50 LD_{50}. It is necessary, however, that all the control mice receiving the test dose of virus die. Because of the nature of the virus and the test involved, it is difficult to obtain exactly the same virus titre from one test to another.

Calculation of Potency [1]

Two principal methods for calculation of potency are employed—gravimetric and volumetric.

1. Gravimetric method

The gravimetric method is designed for use with classical nervous tissue vaccines, e.g., Semple, Fermi, Hempt.

As used here, the ED_{50} (50% effective dose) is the weight in mg of a nervous tissue suspension of rabies vaccine that will protect 50% of mice challenged with a given dose of rabies virus (5–50 LD_{50}, see page 282). The gravimetric method uses the ratio of the ED_{50} of a defined reference vaccine (International Reference Preparation for Rabies Vaccine or an equivalent national reference vaccine) to the ED_{50} of a vaccine under test. This ratio, called the antigenic value (AV), must be at least 0.3 for the vaccine to pass the test.

The *equivalent human dose* of the International Reference Vaccine is considered to be 1 ml of the reconstituted 10% nervous tissue suspension, or 2 ml of a 5% suspension, or any other equivalent quantity of a suspension containing 100 mg of tissue. One human dose of a vaccine under test should therefore contain *at least 100 mg* of tissue, the antigenic value of which must meet the requirements stated above.

Example : The results of the challenge are arranged in columns indicating the number of mice that survived and the number that died. From this tabulation the 50% endpoint dilutions are determined by the method

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Reed & Muench (Appendix 1, Example 2, page 331). If accidental deaths from intercurrent causes result in irregular numbers of inoculated animals per dilution, neither of the methods given in the above examples should be used. Instead the complete formula given on page 327, Example 11, should be applied.

Suppose that the following 50% endpoint dilutions were obtained :

$10^{-1.18}$ for the reference vaccine (example 9, page 323)
$10^{-1.67}$ for the test vaccine.

It is assumed that both vaccines contain 5% of brain tissue (i.e., 50 mg in 1 ml).

The 50% effective dose (ED_{50}) expressed in mg of original brain tissue that will protect 50% of the mice, is calculated for both vaccines using the following formula :

$$ED_{50} = \frac{\text{amount of brain tissue in 1 ml undiluted vaccine}}{\text{reciprocal of 50\% endpoint dilution}}$$

Reference vaccine

$$ED_{50} = \frac{50 \text{ mg}}{10^{1.18}}$$

$$\begin{aligned} \text{or } \log ED_{50} &= \log 50 - 1.18 \\ &= 1.70 - 1.18 = 0.52 \\ ED_{50} &= \text{antilog } 0.52 = 3.31 \text{ mg} \end{aligned}$$

Test vaccine

$$ED_{50} = \frac{50 \text{ mg}}{10^{1.67}}$$

$$\begin{aligned} \text{or } \log ED_{50} &= \log 50 - 1.67 \\ &= 1.70 - 1.67 = 0.03 \\ ED_{50} &= \text{antilog } 0.03 = 1.07 \text{ mg} \end{aligned}$$

The antigenic value (AV) of the brain tissue contained in the test vaccine is obtained by dividing the ED_{50} of the reference vaccine by the ED_{50} of the test vaccine

$$AV = \frac{ED_{50} \text{ of reference vaccine}}{ED_{50} \text{ of test vaccine}} = \frac{3.31}{1.07} = 3.1$$

Since parallel dilutions have been made starting from dilutions containing the same concentration of brain tissue it is easily possible to calculate the relative potency (RP) of a single dose for man of the test vaccine in terms of the reference vaccine. To obtain the RP, the antigenic value is multiplied by a factor that corrects for the difference between the volume of the dose for man of the test vaccine and that of the reference vaccine :

$$RP_{SD} = AV \times \frac{SD_{TV}}{SD_{RV}}$$

where SD = single dose (ml) for man
TV = test vaccine
RV = reference vaccine

It is presumed that 2 ml of the reference vaccine adjusted to 5% brain tissue suspension, represents a single dose for man. Suppose a single dose of the vaccine under test is given as 4 ml, then

$$RP_{SD} = 3.1 \times \frac{4}{2} = 6.2$$

2. Volumetric method

For the more recently developed vaccines prepared from virus suspensions whose nature, concentration, and purity are different from those of classical nervous tissue vaccines the gravimetric NIH-test is inapplicable. This is true of the suckling mouse brain vaccine containing only 1% of nervous tissue (see chapter 23), duck-embryo vaccine prepared from a 33% tissue suspension (see chapter 27), and tissue culture vaccines of which the concentration of the original virus harvest may be about 90% (see chapter 28). For these vaccines the volumetric test should be used. This compares the 50% endpoint *dilution* (highest vaccine dilution protecting 50% of mice) of the vaccine under test with that of the International Reference Vaccine (or equivalent national reference vaccine).

Example :

Unlike the gravimetric method, this method does not require the International Reference Vaccine to be adjusted to the same concentration of virus harvest as the test vaccine. To determine 50% endpoint dilutions the reference vaccine is reconstituted as instructed and serial dilutions are made starting from this suspension, which is considered to contain 10% of brain tissue and of which 1 ml represents a single dose for man. At the same time, parallel serial dilutions of the vaccine under test are made, starting from the prescribed dilution for administration to man.

The relative potency (RP) of the vaccine under test is determined by the formula :

$$RP = \frac{\text{reciprocal of 50\% endpoint dilution of TV}}{\text{reciprocal of 50\% endpoint dilution of RV}} \times \frac{SD_{TV}}{SD_{RV}}$$

Suppose the following 50% endpoint dilutions were obtained :

$10^{-1.18}$ for the reference vaccine (reciprocal $= 10^{1.18}$)

is given as 2 ml and that 1 ml of the reference vaccine represents a single dose for man, then

$$RP = \frac{10^{1.67}}{10^{1.18}} \times \frac{2}{1} = 10^{0.49} \times 2 = \text{antilog } 0.49 \times 2 = 3.1 \times 2 = 6.2$$

If a national reference vaccine is used that differs in potency from the reconstituted International Reference Vaccine, the result obtained with the above formula has to be multiplied by the RP of the national reference vaccine in terms of the International Reference Vaccine.

Minimum Potency Requirements

Where the gravimetric method is used the antigenic value (AV) must be at least 0.3. Manufacturers should be prohibited from increasing the brain tissue concentration and/or the volume of a single vaccine dose should the antigenic value of the brain tissue in the vaccine under test prove to be less than 0.3. A single dose for man must contain at least 100 mg of brain tissue (see above).

Where the volumetric test is used, the RP should be at least 0.3. If the RP of a vaccine under test exceeds this figure the final bulk should *not* be diluted nor should the volume of a single immunizing dose for man be reduced below the figure recommended by the producer at the time of the test.[1]

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[1] Sixth report of the WHO Expert Committee on Rabies (*Wld Hlth Org. techn. Rep. Ser.*, 1973, No. 523). These requirements are under consideration by the WHO Expert Committee on Biological Standardization.

CHAPTER 34

GUINEAPIG POTENCY TEST FOR CHICKEN-EMBRYO VACCINE

H. KOPROWSKI [1]

The test consists of the intramuscular inoculation of guineapigs with chicken-embryo vaccine, followed 3 weeks later by challenge of the animals with street or fixed rabies virus. Animals weighing not less than 350 g at the beginning of the test should be chosen. The use of lighter animals is inadvisable.

Immunization Procedure

Each of 2 vials of vaccine representing each series or subseries in the final containers is reconstituted (irrespective of the manufacturer's recommended dosage) with sterile distilled water containing 2% of normal horse serum (3 ml of the diluent per dog dose of dried vaccine). Combine the 2 doses of the restored vaccine, withdraw a full dog dose (3 ml) and add this to 17 ml of distilled water containing 2% of normal horse serum. This will yield a 5% tissue suspension.

At least 10 healthy guineapigs are identified and inoculated with 0.25 ml of the diluted vaccine, injected into the gastrocnemius muscle on the inside of the leg as near to the nerve as possible. At the same time, 5 or more healthy guineapigs are identified and set aside as vaccine controls.

Preparation of Challenge Material

Street rabies virus

Adult dogs are inoculated with 0.1 ml of a suspension of infected canine submaxillary gland tissue kept frozen at −70°C. The injection is made bilaterally into the masseter muscle, using a 1-ml syringe and a 0.90-mm × 25-mm (20-gauge, 1-in) needle (see figure). When the animals die after showing signs of rabies, or when they are sacrificed when moribund, the submaxillary gland tissue is removed, a small piece is separated by cutting with scissors, and the remainder is frozen in a large Petri dish and kept

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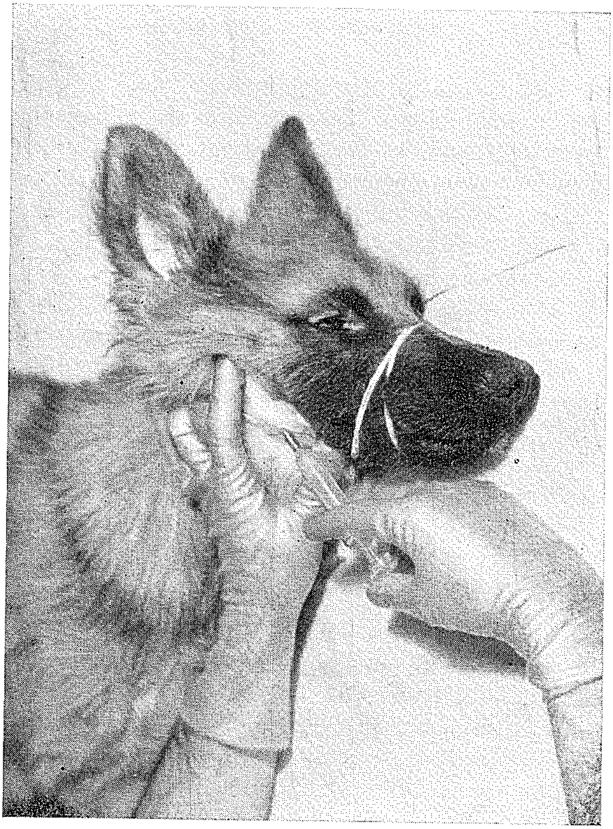
a mortar with enough 10% normal rabbit serum in physiological salt solution to make a 10% suspension by weight. This suspension is then centrifuged in an angle-head centrifuge for one minute at 150–200 *g* and the supernatant liquid is separated. Tenfold dilutions of the supernatant liquid are made, using as diluent 10% normal rabbit serum in physiological salt solution, and 0.03 ml is injected intracerebrally into Swiss albino mice 28–35 days old. These mice are observed for 21 days and the number of dead mice is recorded daily. After the period of observation, the LD_{50} titre of each preparation is calculated (for calculation see Appendix 1, page 321).

In order to make a large virus pool, only those glands with a titre exceeding $10^{-4.5}$ LD_{50} are pooled. The glands are removed from the

INOCULATION INTO MASSETER MUSCLE OF A DOG



By courtesy of Dr F. Perez-Gallardo, Madrid freezer, thawed, cut into small pieces with scissors, and put into a Waring Blendor with enough sterile 10% normal rabbit serum in physiological salt solution to make a 20% suspension by weight. The Blendor is set in motion for 2 or 3 minutes only, in order to prevent heating of the material. It is then immersed in ice-water until well chilled, and the blending is resumed for another 2 or 3 minutes. The procedure is repeated 4 or 5 times, and the final preparation is filtered through one layer of gauze.

The filtered material is distributed in Pyrex-glass ampoules in 1-ml quantities. The ampoules are sealed and the material is shell-frozen in a bath of solid carbon dioxide and ethanol. The ampoules are then stored at a temperature ranging from – 50°C to –70°C.

Fixed rabies virus

The standard challenge virus (CVS) can also be used to challenge guineapigs. The preparation of the pools of virus in mice is described in chapter 33, page 279.

To increase the pathogenicity of this strain for guineapigs by intramuscular inoculation, a few intracerebral passages in young guineapigs (150–200 g) can be performed and a pool of guineapig-brain material used for challenge in place of mouse-brain material.

Challenge of the Guineapigs

Three weeks after vaccination, the vaccinated guineapigs and controls are challenged by an intramuscular injection of canine street or fixed strain of rabies virus.

The dosage used is 0.5 ml of a suitable dilution of the virus suspension injected intramuscularly into the leg opposite that used for vaccination. The dilution is one twofold dilution lower than that which kills 100% of the guineapigs in a preliminary titration ; therefore, if 100% of the guineapigs are killed with the 1 : 160 dilution, a 1 : 80 dilution should be used as the challenge virus for the potency test.

Interpretation of Results

A sample of results obtained with vaccines that have successfully passed potency tests is shown in the accompanying table. It will be seen that the vaccine has proved of definite antigenic value for dogs challenged in this laboratory with street virus. It may also be observed that in most instances dogs were protected against street-virus challenge with a vaccine preparation that immunized guineapigs in 5% chicken-embryo tissue sus-

RESULTS OF POTENCY TEST FOR CHICKEN-EMBRYO VACCINE: MORTALITY RATIOS AFTER INTRAMUSCULAR CHALLENGE WITH STREET VIRUS

Batch of vaccine	LD_{50} titre in mice	Guineapigs							Dogs	
		immunized with the following dilutions of vaccine: [a]						non-vaccinated controls	vaccinated [b]	non-vaccinate controls
		1 : 5	1 : 20	1 : 80	1 : 320	1 : 1280	1 : 5120			
A	$10^{-2.75}$	0/8	0/9	0/6	0/10	0/8	—	10/10	0/5	3/6
B	$10^{-2.85}$	0/9	0/10	5/10	5/10	9/10	—	10/10	0/6 0/3 } two tests performed	3/5 10/10
C	$10^{-2.95}$	0/8	0/8	2/7	6/8	—	—	10/10	0/9 0/6 } two tests performed	8/9 6/10
D	$10^{-3.00}$	0/8	0/6	1/10	4/5	0/9	—	8/8	0/10	10/10
E	$10^{-3.90}$	—	—	1/4	1/6	4/5	0/4	8/9	0/6	18/25
F	$10^{-5.00}$	—	—	0/5	0/3	1/4	1/5	8/9	0/8	18/25
G	$10^{-3.90}$	—	3/15	—	—	—	—	13/16	0/7	3/5

[a] With batches A and D, 1 ml of 5% tissue suspension was used; with all other batches, 0.5 ml was used. (Note 0.25 ml of 5% tissue suspension is now used for routine testing.)

[b] 5 ml of 20% tissue suspension were used for vaccination.

potency to cause the death of most if not all control animals. It has been observed in the past that the titre in mice must be $10^{-5.50}$ LD_{50} or higher for the challenge virus to meet this requirement.

In the actual test, 80% of the control animals should die of rabies within 21 days after challenge with street virus and within 14 days after challenge with fixed virus; 70% of the vaccinated guineapigs, and in any case not less than 7 animals, should survive challenge inoculation without showing any symptoms of rabies. This test very closely parallels events occurring in nature and can be performed with relatively little labour. Only one inoculation with vaccine and one challenge inoculation are required. By employing locally isolated street-virus strain for challenge purposes, the test can be used to evaluate the potency of chicken-embryo vaccines in different geographical areas of the world against different strains of rabies. A disadvantage is that dogs are required for the preparation of challenge virus. However, if a potent preparation of salivary gland tissue is employed, a relatively small number of dogs will suffice to yield a preparation that can be used for a long period for challenge purposes.

Tests with large batches of Flury LEP rabies vaccine in guineapigs, using a CVS virus for challenge of vaccinated animals, have demonstrated that results of potency tests in guineapigs can be correlated with the intracerebral titration of the virus in mice. The paralysis pattern that is observed 4–10 days following the challenge correlates well with the virus titre of the vaccine and can therefore serve to facilitate the selection of potent products.

THE ANTIBODY BINDING TEST FOR VACCINE POTENCY

R. J. ARKO,[1] *T. J. WIKTOR*[2] *& R. K. SIKES*[3]

The antigenicity of an inactivated rabies vaccine of nervous tissue, avian embryo, or tissue culture origin can be determined by measuring its antibody combining activity *in vitro*. Rabies antibody is adsorbed from a standard rabies immune serum on to rabies vaccine in serial dilutions ; the presence of non-adsorbed antibody is then detected *in vivo* by the serum neutralization test in mice or *in vitro* by the plaque-reduction technique. This method reduces the time required to determine the potency values of rabies vaccine from 28 days (Habel and NIH tests) to 14 days when the test is made by inoculation into mice or to 5–6 days when the plaque-reduction technique is used.

Materials and Methods

Reference vaccine. A reference rabies vaccine as used in the NIH test (see Chapter 33, page 280) is employed.

Reference serum. A reference rabies serum containing 10 international units (IU) of rabies antibody per vial can be obtained from WHO reference centres[4] for use in this test (see page 316).

Challenge virus. The standard challenge virus (CVS) strain is prepared and stored as described in Chapter 33, page 279, when the mouse inoculation technique is used. Tissue-culture-adapted CVS or Pitman-Moore (PM) strains are used in the plaque-reduction test.

Mice. White Swiss mice, 3–4 weeks old and uniform in weight, are used.

Tissue culture. Plates of BHK-S13 cells suspended in agarose are prepared as described in Chapter 9, page 109.

Diluent. Phosphate-buffered saline, pH 7.2, containing 2% of inactivated horse serum, is used as a diluent throughout the test.

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[1] Center for Disease Control, Atlanta, Ga., USA.

[2] The Wistar Institute, Philadelphia, Pa., USA.

[3] Assistant Chief, Office of Veterinary Public Health Services, Center for Disease Control, Atlanta, Ga., USA.

[4] A list of WHO reference centres for rabies will be found in Appendix 7, p. 361.

Mouse test procedure

(1) Serial twofold dilutions of the reference and test vaccines are prepared.

(2) The reference serum is diluted to contain 0.1 IU per ml.

(3) 0.5 ml of each vaccine dilution is added to an equal volume of diluted reference serum. These suspensions are mixed and incubated for one hour in a water bath (37°C).

(4) Rabies challenge virus containing 10–50 mouse intracerebral lethal doses (MIC LD_{50}/0.03 ml) is then added in 1-ml portions to each vaccine-serum dilution. The challenge virus is also added to a mixture of vaccine with normal serum to determine the exact amount of virus used in each test.

(5) The vaccine-serum-virus mixtures are incubated for 90 minutes in a water bath (37°C) and then placed in a tray of wet ice.

(6) Six mice are inoculated intracerebrally with 0.03 ml of each vaccine-serum-virus dilution, starting with the tube containing the highest dilution of the vaccine.

(7) The mice are observed for 14 days, the mortality and survival rates are recorded, and the 50% endpoints are calculated.

(8) The effective 50% adsorbing dose of vaccine (AD_{50}) is calculated by determining the dilution of the vaccine required to adsorb sufficient antibody to reduce the antiserum to the point where it will protect only 50% of the mice against the standard dose of challenge virus.

(9) The antigenic value (AV) is determined by dividing the AD_{50} of the test vaccine by that of the reference vaccine (Spearman-Kärber or Reed & Muench method, see Appendix 1, page 321).

Plaque-reduction method

Steps (1), (2), and (3) are the same as for the mouse test procedure described above. Then continue as follows :

(4) After incubation of the vaccine-serum mixture, a tissue-culture-adapted rabies virus (CVS or PM strain) containing 1000 plaque-forming units per ml is added in 1-ml portions to each vaccine-serum dilution.

(5) The vaccine-serum-virus mixtures are incubated for 90 minutes at 37°C.

(6) At least 2 plates of agarose-suspended BHK-S13 cells are inoculated with 0.1 ml of each vaccine-serum-virus dilution.

(7) The plates are incubated for 5–6 days, then stained with an overlay

vaccine-serum-virus mixture is used to determine the 50% plaque-reduction point.

(8) The effective 50% adsorbing dose (AD_{50}) is defined as the vaccine dilution at which the number of plaques is reduced by half. Endpoint determinations are calculated by graphic interpolation (see chapter 9, page 114).

(9) The antigenic value of the test vaccine is derived by dividing the AD_{50} of the test vaccine by that of the reference vaccine.

CHAPTER 36

TEST FOR NEUROALLERGENIC ACTIVITY

R. GISPEN[1]

Principle

Vaccines presumed to be free from neuroallergenic factors should be tested for the absence of encephalomyelitic activity in guineapigs. A suspension consisting of either a fluid vaccine or a reconstituted lyophilized vaccine is mixed with an equal volume of Freund's adjuvant. Guineapigs are inoculated intracutaneously with the mixture. The animals must remain free from central nervous system disease for 40 days. Another group of guineapigs is inoculated with a similar mixture containing normal adult rabbit-brain tissue instead of the vaccine. This group must show signs of encephalomyelitis.

Vaccine

Fluid vaccines are tested in a concentration at least equal to that of the final product.

Lyophilized vaccines are reconstituted with part of the fluid supplied by the manufacturer so as to obtain a concentration equal to 20% of organ tissue or, if the vaccine was produced in cell cultures, equal to the concentration recommended by the manufacturer for immunization.

Normal Brain Suspension

Brain tissue of normal adult rabbits is homogenized mechanically or in a mortar and made up with 0.25% phenol in distilled water to a 20% normal brain suspension (w/v). This suspension may be stored at —25°C.

Adjuvant

The adjuvant consists of 9 ml of Bayol F,[2] 1 ml of Arlacel-A[3] and 40 mg of *Mycobacterium tuberculosis*, H 37 Rv strain killed by autoclaving and lyophilized.

The Arlacel-A is added to the bacteria in a mortar. The bacteria are ground up, after which Bayol F is added and mixed.

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[1] Director, National Institute of Public Health, Utrecht-Bilthoven, Netherlands.

[2] A low-viscosity mineral oil manufactured by Esso Standard Division of Humble Oil & Refining Co., New York, USA.

Equal volumes of vaccines and adjuvant are thoroughly mixed in a mortar so as to obtain a thick emulsion. At intervals a drop of the emulsion is shed on the surface of distilled water in a Petri dish. Both the Petri dish and the water should be changed each time. The emulsion is satisfactory when the drop does not spread over the water.

Equal volumes of normal brain suspension and adjuvant are emulsified in the same way.

Inoculation

At least 10 guineapigs weighing 400–650 g are inoculated with 0.3 ml of the vaccine emulsion by 3 intracutaneous injections of 0.1 ml each on 3 spots of the shorn skin of the neck and proximal part of the back. The animals are observed for 40 days. Animals with signs of paralysis are killed and examined histologically for encephalomyelitis. All animals surviving at the end of the observation period are killed and examined histologically. The guineapigs inoculated with the vaccine must be free from histologically confirmed disease of the central nervous system.

At least 5 out of 10 guineapigs inoculated in the same way with normal brain emulsion must show histological alterations in the brain or spinal cord compatible with experimental "allergic" encephalomyelitis.

PART VI

ANTIRABIES SERUM AND IMMUNOGLOBULIN

CHAPTER 37

PRODUCTION OF ANTIRABIES SERUM OF ANIMAL ORIGIN

P. LÉPINE[1] *& P. ATANASIU*[2]

Pasteur Institute Method

A therapeutic antirabies serum is produced at the Pasteur Institute by immunization of horses or mules with the Pasteur strain of fixed rabies virus. Immunization is begun with β-propiolactone-inactivated vaccine, injection of which is continued for a period of 2 months. This is followed by a series of injections of increasing doses of live virus. All the injections are made subcutaneously. When the volume becomes too large for injection at a single site, multiple injections are made in different parts of the body.

The animals used must be carefully selected, as even for the same breed of horse the suitability of any particular animal for serum production varies according to its age, state of health, nutrition, and history. From time to time, samples of serum are taken from the animals undergoing immunization and the antibody titre is determined. Animals showing an inadequate response are eliminated and only those likely to yield a high-titre serum are retained.

The immunization schedule is as follows :

Day 1 to day 60 : 20 ml of β-propiolactone-inactivated vaccine every other day (30 injections).

Day 61 to day 72 : 4 injections, each of a quarter of a rabbit brain inoculated with fixed virus, at intervals of 3 days.

Day 73 to day 88 : 4 injections, each of half a rabbit brain inoculated with fixed virus, at intervals of 4 days.

Day 89 to day 98 : 2 injections, each of a whole rabbit brain inoculated with fixed virus, with an interval of 5 days.

Day 106 : first bleeding.

Day 136 : 1 injection of a whole rabbit brain inoculated with fixed virus.

Day 144 : second bleeding.

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[1] Pasteur Institute, Paris, France.

is made 8 days later. A booster injection is given after another 30 days, followed by a second bleeding after 8 days. The booster injections and bleedings are repeated at these intervals, but the horses are allowed a rest period of 2 months each year.

Method of the Istituto Sieroterapico e Vaccinogeno, Siena

As originally described by d'Antona & Falchetti in the first edition of this monograph, the Siena method also makes use of horses for the production of a hyperimmune serum. Mirchamsy (1963), at the Razi Institute, Hessarek, Iran, slightly modified the method for the immunization of adult mules.

The vaccine used by Mirchamsy is prepared from sheep inoculated intracerebrally with Sassari fixed virus and consists of a 5% brain-tissue suspension containing 0.5% of phenol and inactivated at 37°C for 24–48 hours. The mules used for production of the serum are simultaneously immunized against tetanus by several injections of tetanus toxoid adsorbed on aluminium phosphate (PATT).

The immunization schedule is as follows :

Day 1 : 20 ml of phenolized vaccine + 10 ml of PATT.
Day 2 to day 20 : 20 ml of phenolized vaccine daily (19 injections).
Day 21 : 30 ml of phenolized vaccine + 25 ml of PATT.
Day 22 to day 40 : 30 ml of phenolized vaccine daily (19 injections).
Day 41 : 45 ml of phenolized vaccine + 40 ml of PATT.
Day 42 to day 56 : 45 ml of phenolized vaccine daily (15 injections).
Day 57 to day 115 : resting period.
Day 116 : 30 ml of phenolized vaccine + 30 ml of PATT.
Day 117 to day 126 : 30 ml of phenolized vaccine daily (10 injections).
Day 127 : 45 ml of phenolized vaccine + 45 ml of PATT.
Day 128 to day 136 : 45 ml of phenolized vaccine daily (9 injections).
Day 137 : 30 ml of a 5% suspension of live virus (Sassari fixed strain) + 60 ml of PATT.
Day 138 to day 144 : 30 ml of a 5% suspension of live Sassari virus daily (6 injections).
Day 156 : first bleeding.

Accelerated Method

Fuenzalida & Palacios (1964) developed an accelerated method of hyperimmunizing horses. The animals are first given a series of subcutaneous injections of vaccine in increasing concentrations and then simultaneous subcutaneous, intraperitoneal and intradermal injections of pure virus suspended in Freund's adjuvant without mycobacteria.

The horses are bled 20 days after the completion of the immunization schedule, which is as follows :

Day 1 : 40 ml of 1% inactivated vaccine subcutaneously.
Day 7 : 40 ml of 2% inactivated vaccine subcutaneously.
Day 14 : 40 ml of 5% inactivated vaccine subcutaneously.
Day 34 : 3 injections of an 8% suspension of live virus (brain tissue) in Freund's adjuvant — 5 ml intraperitoneally, 4 ml subcutaneously, 1 ml intradermally.
Day 41 : 3 injections of 16% live virus suspension in Freund's adjuvant — 5 ml intraperitoneally, 4 ml subcutaneously, 1 ml intradermally.
Day 48 : 3 injections of 20% live virus suspension in Freund's adjuvant — 8 ml intraperitoneally, 6 ml subcutaneously, 1 ml intradermally.
Day 68 : bleeding.

The vaccine used for the initial stages of immunization is prepared by the method previously described by Fuenzalida & Palacios (1955). Suckling mice are inoculated with fixed rabies virus and the vaccine prepared from the brain tissue is inactivated by irradiation with ultraviolet light. The live-virus suspensions are also prepared from the brains of suckling mice, with the addition of Freund's adjuvant without mycobacteria.

This rapid method seems to produce very satisfactory immunization and Fuenzalida & Palacios report neutralizing antibody titres that compare favourably with those obtained by other methods of immunization.

Immunization of Equines with Cultured Rabies Antigen

Antigens used

Two types of antigen—live and inactivated—are used. Both are prepared from the Pasteur strain of rabies virus cultured on BHK-S13 cells and maintained by weekly passages.

(*a*) *Inactivated antigen*

Cultures from the 20th passage are pooled to give a total volume of about 10 litres. 0.03 ml of this pool usually gives a titre of $10^{-5.1}$ in the mouse and $10^{-5.5}$ in cell cultures.

The antigen is inactivated with β-propiolactone, using 0.1 ml for 1000 ml of antigen ; inactivation is confirmed by intracerebral inoculation

Cultures from the 20th passage are pooled with an equal volume of cultures from the 23rd passage. 0.03 ml of this pool usually gives a titre of $10^{-5.1}$ in the mouse and $10^{-5.5}$ in cell cultures.

Both antigens are stored in the frozen state at —30°C.

Immunization schedule

Either horses or mules may be used for immunization. Before commencing the immunization schedule, the animals are bled to test for possible cytopathogens in the serum. Provided that no cytopathogenic activity is demonstrated, immunization is carried out as follows :

First stage, using inactivated antigen

(*a*) Weekly intramuscular injections for a period of 3 weeks with the following doses :

1st injection	20 ml
2nd injection	50 ml
3rd injection	100 ml
4th injection	100 ml.

(*b*) Two intramuscular injections of 100 ml weekly for 3 weeks, i.e., a total of 6 injections, followed by the first control bleeding.

(*c*) Weekly intramuscular injections of 100 ml for 2 months, i.e., a total of 8 injections, followed by the second control bleeding.

Second stage, using live antigen

Injections once a week of the following doses :

1st injection	20 ml
2nd injection	50 ml
3rd injection	100 ml
4th injection	100 ml

followed by the third control bleeding.

This schedule is continued, with weekly injections of 100 ml and periodic bleedings, until a satisfactory titre is achieved.

Determination of protective titre

At each control bleeding, a steady rise in the antibody titre should be observed. Present experience indicates that the following titres can be expected at the first 3 bleedings.

First bleeding : The protective titre determined on cell cultures against 100 LD_{50} of virus should reach approximately 1 : 300, corresponding to about 30 IU/ml.

Second bleeding : The protective titre is determined in mice in comparison with the International Standard containing 86.6 IU of rabies antibody per vial.[1] Using 100 LD_{50} of standard challenge virus (CVS), the reference serum should give a titre of about 1 : 2800 and the serum under test a titre above 1 : 3000, corresponding to more than 90 IU/ml. Using 300 LD_{50} of CVS, the reference serum should give a titre of approximately 1 : 1300 and the serum under test a titre of 1 : 2800, corresponding to about 170 IU/ml. When the titre is determined in cell cultures using 300 LD_{50} of virus, a value above 1 : 3000 should be obtained.

Third bleeding : The protective titre determined in mice infected with 300 LD_{50} of CVS should be about 1 : 6500, or 450 IU/ml.

Concentration and Purification of Antirabies Serum

The relatively large amounts of antirabies serum necessary for the protection of exposed persons, as well as the risk of anaphylactic accidents and other reactions, have led to the development of a number of methods for preparing a purified, concentrated serum.

Protein fractionation was first attempted by Habel (1945) using ammonium sulfate, and various other methods have since been described.[2] The method of fractionation and purification adopted at the Pasteur Institute consists of 2 stages : first, enzymatic digestion of the proteins followed by precipitation with ammonium sulfate ; second, removal of the excess proteins by thermocoagulation. (See also chapters 38, 39 and 40.)

Whichever method is adopted, it is advisable to determine the final protein content of the purified serum and relate this to its protective titre (see chapter 40, page 314). Paper electrophoresis should also be performed to check the fractionation of the proteins. In general, a concentrated purified serum with a titre of 120 IU/ml should not contain more than 5% of total serum proteins.

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[1] See chapter 40, pages 316 & 317. A reference serum may be obtained from WHO reference centres for rabies (see Appendix 7).

PREPARATION OF ANTIRABIES IMMUNOGLOBULIN OF ANIMAL ORIGIN: METHOD USED IN THE USSR

M. SELIMOV[1] *& E. GORDIENKO*[2]

Antirabies immunoglobulin is manufactured in the USSR from the hyperimmune serum of horses immunized with a 10% sheep-brain suspension of fixed rabies virus (Moscow strain, a variant of the Pasteur strain). The vaccine is of the Fermi-type and contains 0.5% of phenol. It is partially inactivated by keeping at 22°C for 8 days and is then injected into the subcutaneous tissue in various parts of the body. The schedule for immunization of the animals used for globulin production is set out in the following table :

IMMUNIZATION SCHEDULE FOR HORSES USED IN THE PRODUCTION OF ANTIRABIES IMMUNOGLOBULIN

Immunization stage	Day	Antigen dose
1	1	40 ml
	7	80 ml
	14	120 ml
2	21	50 ml
	28	100 ml
	35	150 ml
3	42	50 ml
	49	100 ml
	56	150 ml
	63	Test bleeding
4	73	150 ml
	80	Test bleeding
5	96	150 ml
	103	Bleeding

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[1] Professor and Head, Rabies Prophylaxis Laboratory, Institute of Poliomyelitis and Viral Encephalitic Diseases, USSR Academy of Medical Sciences, Moscow, USSR.

[2] Head, Rabies Vaccine Section, Kharkov Scientific Research Institute for Vaccines and Sera, Kharkov, USSR.

When the titre of virus-neutralizing antibodies exceeds 1 : 500 (measured against 100 LD_{50} of fixed rabies virus) the animals are given an injection of a single dose of 150 ml of antigen. After the 5th stage of immunization, horses with an antibody titre of less than 1 : 500 are either immunized intramuscularly (10, 20 or 30 ml of the antigen at weekly intervals) or are not used for immunoglobulin production.

Extraction of the Immunoglobulin Fraction

A mixture of crude antirabies serum with an antibody titre of at least 1 : 1000 measured against an average of 100 LD_{50} of fixed rabies serum (CVS strain) is precipitated with ethanol in the cold by a modification of Cohn's method (1961).

First stage

The serum is diluted with an equal volume of distilled water and agitated in the cold for 2 hours ; 352 ml of 96% ethanol are added per litre of the mixture at a rate of 5–6 litres per hour (final concentration of the ethanol : 25%). The precipitate is separated by centrifugation at −5°C.

Second stage

The precipitate is dissolved in distilled water (25 litres per kg of precipitate). 10 ml of 1M sodium chloride solution are added to each litre of the mixture, and the pH is adjusted with acetate buffer to 5.0 ± 0.1. The mixture is then agitated for 3 hours, after which 96% ethanol is added in the proportion of 215 ml per litre of the mixture (final concentration of the ethanol : 17% ; temperature of the mixture 0–6°C). After agitation for an hour the mixture is separated in an ASG-3M separator[1] at a rate of 20 litres per hour. The temperature of the mixture on leaving the separator is 4.5–5°C. The centrifugate should be clear.

Third stage

The centrifugate is poured into a reaction vessel and the following are added :

1M sodium chloride solution, 50 ml per litre of centrifugate ;

1M sodium bicarbonate solution, 5 ml per litre of centrifugate, pH 7.0–7.2, with continuous mixing ;

34% ethanol, in an amount equal to the combined volume of the sodium chloride and sodium bicarbonate solutions ;

96% ethanol, in the proportion of 113 ml per litre of centrifugate, to give a final concentration of 25%.

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for 1 hour the mixture is separated at the rate of 25 litres per hour. One litre of crude antirabies serum yields 43 g of antirabies immunoglobulin precipitate.

In order to remove the ethanol, the crude immunoglobulin precipitate is placed in sterile muslin and then in filter cloth, and filtered under mechanical pressure for 24 hours at 8–10°C. The crude precipitate is used to prepare a 10% ± 1% solution of immunoglobulin in physiological saline (pH 7.0–7.5). Within 24 hours of preparation, the immunoglobulin solution is filtered under pressure through Seitz-type (Sal'nikov) filters fitted with sterilizing plates with a pore diameter of 300 µm.

To achieve stabilization, the immunoglobulin solution is kept for 20–30 days at room temperature and then filtered again.

Control tests of the antirabies immunoglobulin are made at all stages of production. The final preparation is tested for :

(*a*) freedom from bacterial and fungal contaminants ;

(*b*) safety ;

(*c*) protective activity (the titre should be not less than 580 IU per ml by comparison with the WHO reference serum[1]) ;

(*d*) electrophoretic homogeneity in a Tiselius apparatus or on paper (immunoglobulin content not less than 97%) ;

(*e*) protein concentration (10% ± 1%) ;

(*f*) residual ethanol (not exceeding 4.5%) ;

(*g*) turbidity (the preparation should be transparent and should not exhibit more than slight opalescence).

The antirabies immunoglobulin is stored at 2–4°C. The expiry date is 2 years from the time at which the protective activity was determined.

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[1] Obtainable from WHO reference centres for rabies serum (see Appendix 7). See also footnote on page 317.

CHAPTER 39

PREPARATION OF ANTIRABIES IMMUNOGLOBULIN OF HUMAN ORIGIN

R. K. SIKES[1]

The use of homologous immunoglobulins for human postexposure treatment virtually eliminates the risk of anaphylaxis and serum sickness normally associated with heterologous serum products. Approximately 16% of persons treated with antirabies serum of equine origin develop serum sickness. For persons over 15 years of age, the incidence increases to 46% (Karliner & Belaval, 1965).

Efforts have therefore been directed to the production of rabies immunoglobulins of human origin. So far, several lots prepared on a large scale have proved statistically to be as effective as antirabies horse serum in preventing rabies in experimental animals (Winkler et al., 1969 ; Sikes, 1969).

This chapter contains the detailed procedures for the preparation of immunoglobulins of human origin, as applied at the Center for Disease Control, Atlanta, USA.

Two major problems must be overcome :

(1) a supply of donors whose antibody titre is at least 1 : 400 must be obtained ;

(2) the cost of providing such a specialized globulin is still very high.

Formula

Human rabies immune globulin (HRIG) for intramuscular administration is a 16.5% ± 1.5% solution of immunoglobulin in 0.3 M glycine, and preserved with a 1 : 10 000 thiomersal[2] solution.

Source and Shipment of Blood

Donors of plasma for the production of HRIG should have demonstrated high levels of neutralizing antibody following pre-exposure or postexposure immunization with a rabies vaccine. One or more booster immunizations with a licensed rabies vaccine should be given 1–2 weeks before the first collection of blood. In order to prepare HRIG of sufficient potency, and assumnecessary to start with a rabies immune plasma pool containing 8–10 antibody units per ml or having a serum neutralizing antibody titre of 1 : 700 to 1 : 1000. Studies have shown that, following a booster dose of antirabies vaccine, about 10% of subjects who have received pre-exposure antirabies prophylaxis and about 40% of those with a history of post-exposure treatment will develop antibody titres of sufficient levels.

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[1] Veterinary Director, Office of Veterinary Public Health Services, Center for Disease Control, Atlanta, Ga., USA.

The collecting centre may obtain a unit of plasma by plasmapheresis or by separation of a unit of whole blood aseptically. The plasma is shipped refrigerated to the laboratory and is stored frozen until it is ready to be pooled for fractionation.

Reagents

The preparation and storage of the reagents required are described in the Annex on page 312.

Technique[1]

The immunoglobulins are separated from the plasma by the cold ethanol fractionation technique described below (Cohn et al., 1946).

Precipitation of fraction I

1. Pool the plasma ; pour individual units through cheesecloth into a tared vat large enough to contain 1.2 litres per litre of plasma.

2. Determine the pH ; if necessary, adjust to the range 7.0–7.4 with acetate buffer (80-fold concentrate) or 0.5M sodium phosphate solution.

3. Freeze three 15-ml samples of pooled plasma.

4. Determine the weight of plasma.

5. Cool the plasma to 0°C and add 163 g of cold 53.3% ethanol per kg of plasma. Adjust the rate so that the addition of ethanol takes about 1 hour. Cool to −2°C during the addition. Stir for half an hour after the addition of ethanol is completed.

6. Centrifuge into a tared vat large enough to contain 1.7 litres per litre of original plasma. Maintain the temperature at −2°C to −3°C during centrifugation. Freeze a 15-ml sample of supernatant I (S1) and weigh it. Maintain at −2°C, and begin precipitation of fraction II-III.

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[1] The procedures used in this chapter are used by the Bureau of Laboratories, Michigan Department of Public Health, East Lansing, Mich., USA ; they have been adopted for the fractionation of HRIG at the Center for Disease Control, Atlanta.

Precipitation of fraction II-III (P2)

1. For every kg of supernatant I, add 552 g of 53.3% ethanol to which has been added 1.33 ml of acetate buffer 80-fold concentrate. Adjust the rate so that the addition of the ethanol takes about 1 hour. Cool to −9°C during the addition.

2. Stir at −9°C for half an hour after the addition of ethanol is completed.

3. Centrifuge, collecting the centrifugate in a tared bowl. Maintain the temperature at −6°C to −9°C during centrifugation.

4. Determine the weight of the precipitate, which is fraction II-III (P2). Freeze in the bowl at −20°C, and hold for precipitation of fraction II-IIIw.

Precipitation of fraction II-IIIw (P2w)

1. Remove frozen P2 from the centrifuge bowl and rapidly homogenize to a uniform suspension in a mixture of water and crushed ice. Use 2 g of water-and-ice mixture per gram of P2. Avoid excessive foaming.

2. For every gram of P2 immediately add a mixture containing 0.107 ml of 0.5M sodium hydrogen phosphate (Na_2HPO_4) in 2.89 g of water and crushed ice.

3. Stir in the cold until all the solid is in suspension.

4. Pour into a vat containing 20 g of cold water per gram of P2, and stir at 1°C for 30 minutes or until no ice remains in the mixture. The vat should be large enough to contain 4 litres per 100 g of P2.

5. Remove 5 ml of the mixture from the vat, add 5 ml of 0.15M sodium chloride solution, and determine the pH; if necessary, adjust the pH of the vat mixture to between 7.0 and 7.4 by addition of acetate buffer 80-fold concentrate or 0.5M sodium hydrogen phosphate solution.

6. Freeze a 15-ml sample.

7. Add 14.1 g of 53.3% ethanol per gram of P2; adjust the rate so that the addition of the ethanol requires about 1 hour. Cool to −6°C during addition of the ethanol. Stir at −6°C for 2–4 hours after the addition of ethanol is completed.

8. Centrifuge and collect the centrifugate in a tared bowl. Maintain the temperature at −5°C to −7°C during centrifugation.

9. Freeze a 15-ml sample of supernatant II-IIIw (S2w).

10. Determine the weight of precipitate, which is fraction II-IIIw (P2w). Freeze in the bowl at −20°C and hold for precipitation of fraction

1. Remove the frozen P2w from the bowl, and rapidly homogenize to a uniform suspension in a mixture of crushed ice and water. Use 2 g of water-and-ice mixture per gram of P2w. Avoid excessive foaming.

2. Immediately add 2 ml of cold 0.175M sodium acetate solution per gram of P2w, and stir in the cold until all the solid is in suspension.

3. Adjust the pH to 5.2 ± 0.1 by addition of acetate buffer 80-fold concentrate diluted 1 : 25 with cold water, and then add more cold water until the total amount added is 1 ml per gram of P2w.

4. Stir in the cold for 1 hour or until no ice remains in the mixture. Determine the pH and adjust, if necessary, to the range 5.1–5.3.

5. Pour into a vat containing 13.5 g of cold water per gram of P2w. The vat should be large enough to contain 2.9 litres per 100 g of P2w.

6. Add 8.1 g of 53.5% ethanol per gram of P2w; adjust the rate so that the addition of ethanol requires about 1 hour. Cool to −6°C during the addition of the ethanol.

7. Stir at −6°C for half an hour after the addition of ethanol is completed.

8. Centrifuge into a cold pressure tank and filter into a tared vat. The vat should be large enough to contain 3.1 litres per 100 g of P2w. Use a 1.2 µm "Versapor" pre-filter[1] over a 0.8-µm membrane. Maintain the temperature at −5°C to −6°C during centrifugation and filtration.

9. Freeze a 15-ml sample of supernatant III (S3).

10. Determine the weight of S3, maintain at −6°C, and proceed with the precipitation of fraction II.

Precipitation of fraction II

1. Stir S3 vigorously at −6°C, and for every kg of this solution slowly add 2 g of sodium hydrogen carbonate.

2. Add 2 ml of the vat mixture to 8 ml of 0.15M saline, and determine the pH; if necessary, add additional sodium hydrogen carbonate to adjust the pH to the range 7.4 ± 0.2.

3. Add 94.7 g of 95% ethanol per kg of solution. Adjust the rate so that the addition of ethanol requires about 1 hour. Cool to −9°C during the addition of the ethanol.

4. Centrifuge, collecting the centrifugate in a tared bowl. Maintain the temperature at −6°C to −9°C during centrifugation.

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[1] An epoxy reinforced glass filter obtainable from Gelman Instrument Co., Ann Arbor, Mich., USA. Type G pre-filters (see next page) are available from the same source.

5. Determine the weight of fraction II, freeze in the bowl at −20°C, and hold for lyophilization.

Fraction II lyophilization and final preparation

1. Remove the frozen fraction II from the bowl, and rapidly homogenize to a uniform suspension in cold 0.3M glycine, using 1 ml per gram of frozen paste.

2. Add 4 g of cold water[1] per gram of paste; stir for 2–4 hours in the cold, and allow to stand overnight at 0°C without stirring.

3. Decant the supernatant, lyophilize, and determine the weight of lyophilized product.

4. For preparation of the final solution of the product, the quantities are calculated as follows:

(*a*) Dry weight of powder = weight of lyophilized powder (g) $\times 0.98$.

(*b*) Volume of powder (ml) = $a \times 0.75$.

(*c*) Volume of water to be added (ml)
= ml of glycine added before lyophilization $-b$.

(*d*) Dry weight of globulin (g)
= $a - (0.0225 \times$ ml glycine added before lyophilization).

(*e*) Final volume (ml) $= \dfrac{d}{0.17}$

(*f*) Volume of 0.3M glycine to be added (ml) $= e - b - c$

(*g*) Volume of 10% thiomersal to be added (ml) $e \times 10^{-3}$.

5. Put the required volume of water (*c*)[1] and 0.3M glycine (*f*) in a beaker with a magnetic stirring bar, and add the lyophilized globulin. Stir to solution; avoid excessive foaming.

6. Add the required volume of 10% thiomersal (*g*). A second person should check this calculation and observe the addition. Determine the pH and adjust, if necessary, to 6.8 with acetate buffer 80-fold concentrate.

7. Filter the globulin solution through a 0.8 μm membrane with 1.2 μm "Versapor" and glass type G pre-filters.[2]

8. In the sterile room, sterilize the globulin solution by filtration into the bulk product container.

9. Perform the bulk container sterility test with fluid thioglycolate medium. Use one 2-ml sample.

[illegible]

tainer filling.

Testing

Tests for safety and sterility, pyrogenic substances, heat stability, pH, and turbidity are needed to ensure that the product satisfies the minimum requirements specified in national standards. For potency requirements see chapter 40, p. 314.

Annex

PREPARATION AND STORAGE OF REAGENTS

Unless otherwise specified, reagent grade chemicals are used.

Distilled water

Water is collected from the still directly into a pressure-filtration tank and filtered through a sterile 0.2-µm membrane into a sterile dispensing vessel. Except when pyrogen-free Water for Injection [1] is required, this water is used in all procedures and for making ice and reagents.

95% (v/v) ethanol

95% ethanol is filtered through a 0.2-µm membrane (not sterile) into dispensing vessels and stored under refrigeration (cold room).

53.3% (v/v) ethanol

5060 g of water are added to 4940 g of 95% ethanol. The mixture is filtered through a 0.2-µm membrane (not sterile) and stored under refrigeration (cold room). The specific gravity, measured with a 60°F/60°F hydrometer at 15°C, should be between 0.9275 and 0.9285.

Acetate buffer, 80-fold concentrate

Dissolve 108.9 g of sodium acetate and 240.2 g of glacial acetic acid in water and make up to one litre. This yields an 80-fold concentrate. When this concentrate is diluted with 80 parts of water the pH of the buffer should be 4.00 ± 0.02.

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[1] See *Specifications for the quality control of pharmaceutical preparations—second edition of the International Pharmacopoeia*, Geneva, World Health Organization, 1967, p. 50.

0.5M sodium phosphate

Dissolve 71.0 g of sodium hydrogen phosphate (Na_2HPO_4) or 89.1 g of the dihydrate (Na_2HPO_4, $2H_2O$) in water and make up to one litre. The pH of this solution should be 9.2. Store at room temperature.

0.175M sodium acetate

Dissolve 23.8 g of sodium acetate in water and make up to one litre.

0.3M glycine

Only glycine that has been determined to be pyrogen-free should be used. A 0.3M solution contains 22.5 g of glycine per litre. Use Water for Injection[1] to prepare this solution. It should always be freshly prepared for use.

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QUANTITATIVE ASSAY AND POTENCY TEST OF ANTIRABIES SERUM AND IMMUNOGLOBULIN

P. ATANASIU[1]

Principle

The method described below consists in neutralizing a constant dose of the previously titrated challenge virus with a series of different dilutions of the serum. The method is used mainly for the assay and potency testing of therapeutic antirabies serum and immunoglobulin, but it is also applicable to any serum containing rabies antibody. Thus, it can be used to determine the antibody titres of human sera collected during therapeutic trials of different vaccines.

The method comprises the following 3 stages:

1. Preparation and titration of the challenge virus.
2. Serum-virus neutralization—preparation of the serum and of the serum-virus mixtures; inoculation of mice.
3. Interpretation of the results.

The serum neutralization assay by means of the plaque reduction technique is described in chapter 9, page 113.

1. Preparation and Titration of Challenge Virus

Challenge virus

The strain normally used is the standard challenge virus strain (CVS), as used in the mouse potency test for rabies vaccine (see chapter 33, page 279). The laboratory strain of fixed virus may also be used, provided that its LD_{50} for the mouse is known and remains constant.

Titration of stock virus

The challenge virus is stored as a 20% suspension, which is dispensed into ampoules and deep frozen. When carrying out the test, an ampoule is taken from the stock and thawed rapidly under the tap. Serial tenfold

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[1] Head, Rabies and Rhabdovirus Research Laboratory, Pasteur Institute, Paris, France.

dilutions are then prepared, giving concentrations of 2×10^{-2}, 2×10^{-3} etc., up to 2×10^{-7}. The diluent used is double-distilled water, to which has been added 2% of normal horse serum, inactivated for 30 minutes at 56°C.

In each of 5 haemolysis tubes is then placed 0.5 ml of one of the virus dilutions, starting with 2×10^{-3} and ending with 2×10^{-7}. To each tube is then added 0.5 ml of inactivated horse serum diluted 1 : 5, so that the final dilutions in the 5 tubes are 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}.

After shaking, the mixtures are incubated for $1\frac{1}{2}$ hours at 37°C, in order to ensure that the conditions are the same as those of the serum-virus neutralization stage. The tubes are then cooled in ice-water. Mice are inoculated intracerebrally with 0.03 ml of the virus suspension, 5 mice being used for each dilution. Provided that the highest dilution is used first, all the inoculations may be made with the same syringe. A record is made of the number of mice that die between the 6th and 20th day after inoculation.

For the calculation of the virus titre, see Appendix 1, Table 1 (page 323), examples 1–3.

Calculation of challenge doses

As an example of how challenge doses are calculated, let it be assumed that a given CVS stock preparation contains $10^{-5.84}$ LD_{50} per 0.03 ml, i.e., 1 LD_{50} is contained in 0.03 ml of a $10^{-5.84}$ dilution of the stock virus. For sera of high titre, between 100 and 300 LD_{50} are used in the serum-virus neutralization assay. To find the dilution of CVS that will contain 300 LD_{50} in 0.03 ml the logarithm of 300 is subtracted from the logarithm of the assumed titre of the stock preparation :[1]

log dilution of challenge preparation containing 300 LD_{50} of virus per 0.03 ml $= \log 10^{-5.84} - \log 300 = 5.84 - 2.48 = 3.36$

Since $3.36 = \log 2300$, this means that the CVS stock preparation will have to be diluted 1 : 2300. For this purpose, first make serial tenfold dilutions of the stock CVS until a 10^{-3} dilution is obtained, then add 1.3 ml of diluent for every 1 ml of the 10^{-3} dilution. The final dilution will then contain 300 LD_{50} of virus per 0.03 ml.

For sera of low titre (e.g., postvaccinal human sera), only 50 LD_{50} may be needed. The calculation then becomes :

$$\log 10^{-5.84} - \log 50 = 5.84 - 1.70 = 4.14$$

Since $4.14 = \log 14\,000$, the CVS stock preparation will have to be diluted 1 : 14 000 by making tenfold dilutions down to a dilution of 10^{-4} and then adding a further 0.4 ml of diluent for every 1 ml of this dilution. This gives a final dilution containing 50 LD_{50} of virus per 0.03 ml.

Inactivation

The sera to be tested are inactivated for 30 minutes at 56°C.

Neutralization

The following serial dilutions of the serum or immunoglobulin under test are prepared : 1 : 500, 1 : 1000, 1 : 2000, 1 : 4000, etc., so that the final dilution will show no neutralization.[1] From each of these dilutions, 0.5 ml is transferred to one of a series of test tubes. Next, 0.5 ml of the virus dilution corresponding to 300 LD_{50} (in the example given above, a dilution of 1 : 2300) is added to each tube. This results in a twofold dilution of both the virus and the serum, so that the final virus dilution is 1 : 4600, or $10^{-3.66}$, and the final serum dilutions are 1 : 1000, 1 : 2000, 1 : 4000, 1 : 8000, etc.

It is advisable to include in the test a reference serum, which is titrated at the same time as the unknown sera. This reference serum is a serum of known titre that has previously been titrated against the International Standard[2] and then stored in the laboratory with the usual precautions. If the results of the test are to be expressed in international units (IU), the inclusion of such a reference serum is mandatory.

Titration of virus control

It is essential to determine the actual quantity of virus used in the test. For this purpose, 0.5 ml of the challenge virus (dilution 1 : 2300 = 300 LD_{50} per 0.03 ml) is mixed with 0.5 ml of diluent containing 20% of inactivated normal horse serum. Thus, a dilution of challenge virus is obtained that contains 150 LD_{50} per 0.03 ml. This tube is marked "O" and 3 serial tenfold dilutions are prepared and marked −1, −2, and −3. After shaking, the tubes containing the sera under test and the control tubes are incubated at 37°C for 1½ hours.

Inoculation of mice

After incubation at 37°C for 1½ hours, all the tubes are placed in a vessel filled with ice-water. Batches of mice weighing 14–16 g each are then inoculated intracerebrally with 0.03 ml of each dilution, using 5 mice per dilution. This is done both for the sera under test and for the virus control. When a large number of sera are being tested, it is important to make the injections of the virus control after having injected half the sera ;

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[1] If the levels of antibody are expected to be low, as in vaccinated human subjects, serum dilutions starting with 1 : 5, 1 : 25, 1 : 125 are employed and the final virus dilution should be 20–50 LD_{50}.

[2] See footnote on next page.

this ensures that the dilutions of the virus control and the sera under test are kept for the same average length of time before making the injections. The different groups of mice are placed separately in labelled jars and kept under observation; a record is made of those dying between the 6th and 20th day.

3. Calculation and Interpretation of Results

The following example is based on the titration of an experimental batch of therapeutic serum with dilutions of the stock virus referred to in the example on page 315.

Determination of LD_{50} actually used in the test

Tube	*Deaths*	*Survivals*
0	5	0
−1	5	0
−2	4	1
−3	0	5

Applying the same calculation as in Example 2 in Appendix 1 (Table 1, page 323) and Table 5, page 326), the 50% endpoint dilution of the virus is found to be $10^{-2.3}$. The antilog of 2.3 (= 200) is the actual number of LD_{50} used in the reaction mixtures for neutralization.

Calculation of the ED_{50} of the serum under test

This calculation is illustrated in Appendix 1 by Example 7, Table 2, page 323; dilution factor = 1 : 2. In this case, the total number of survivors is determined and the 50% endpoint dilution is estimated by using Table 3 (page 324). For the serum under test the ED_{50} is found to be $10^{-3.21}$ or 1 : 1620. The ED_{50} of the reference serum is similarly estimated from the tables.

Example 8 in Appendix 1, page 323, illustrates the calculation of the ED_{50} for a low-titre serum diluted 1 : 5, 1 : 25, 1 : 125, and 1 : 625. This range of dilutions is frequently used for the postvaccinal antibody assay of human sera (see footnote on p. 316). In the example used the ED_{50} (or antibody titre) found is $10^{-2.10}$ or 1 : 125.

The number of international units (IU) contained in the International Standard for Antirabies Serum is fixed arbitrarily at 80 per ml.[1] To express compared with that of the International Standard or with that of a reference serum that has been calibrated against the International Standard. This is done by calculating the difference between the logarithms of the 50% endpoint dilutions of the two sera. In example 7 referred to above, the 50% endpoint dilution of the serum under test is $10^{-3.21}$. Assuming the 50% endpoint dilution of the reference serum to be $10^{-3.19}$, the calculation becomes :

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[1] This is a round figure in order to simplify the calculations. Actually, the International Standard for Antirabies Serum contains 86.6 mg of dried serum per ampoule, which is equivalent to 86.6 IU. This serum is available to national laboratories, on request, from the Inter-

$$-3.19-(-3.21) = 0.02.$$

The serum under test is therefore $10^{0.02} = 1.05$ times as potent as the reference serum, so that its potency is $1.05 \times 80 = 84$ IU per ml.

Therapeutic potency test

An antirabies serum should satisfy the requirements laid down in the fourth report of the WHO Expert Committee on Rabies :[1]

> A serum shall pass the test for sufficient therapeutic potency if, in a single comparative assay, it is revealed to be equal to or better than the International Standard Serum. In case a serum fails the test, two more similar tests may be carried out. If the serum proves equal to or better than the International Standard Serum in both these additional tests, it shall pass. The outcome is " equal " or " better " if the total survivor fraction (survivors/total number of mice) for the serum under test is equal to or larger than that for the standard.

These requirements are also applicable to immunoglobulin.

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1960, **201**, 10.

PART VII

APPENDICES

APPENDIX 1

METHODS OF CALCULATION

R. J. LORENZ[1] *& K. BÖGEL*[2]

A. THE SPEARMAN-KÄRBER METHOD

This method is based on an idea first put forward by Spearman (1908) and later developed by Kärber (1931). Further refinements were described by Finney (1952). The calculation of 50% endpoint dilutions using the Spearman-Kärber formula is described on pages 327–329. A great advantage of the method, however, is that this rather lengthy procedure can be circumvented by the use of simple tables, provided that no accidental deaths among the experimental animals have to be taken into account (see below). Since the method also permits a relatively high degree of accuracy, it is being increasingly used in virology.[3]

Procedure using Tables

According to the formula given on page 327 each individual positive indicator (mouse) increases the logarithm of the 50% endpoint by a constant numerical value, provided that the dilution factor and the number of animals inoculated per dilution are both constant.

The $\log_{10}$ of the 50% endpoint dilution is therefore obtained by simple addition of two values (Tables 1 and 2), the first derived from the lowest dilution of virus, serum, or vaccine at which all animals are positive, the second related to the total number of positive animals ("positives") at this dilution and at all higher dilutions. The second value is read off from Tables 3–5 (pages 324–326) which have been prepared for different dilution factors and numbers (n) of animals inoculated per dilution.

This procedure also permits a quick evaluation of the possible error due to accidental deaths. In a first calculation accidental deaths are considered as "positives" and in a second one as "negatives". If either titre has a bearing on the interpretation of the result (as in the case of marginal values obtained in potency tests), the test has to be repeated (see also "Special considerations" on page 328).

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[1] Federal Institute for Animal Virus Diseases, Tübingen, Federal Republic of Germany.

[2] Veterinary Public Health, Division of Communicable Diseases, World Health Organization, Geneva, Switzerland.

[3] Some virologists may still prefer to use the method of Reed & Muench, given in previous

The numerical values attributed to "positives" for different dilution factors and different values of *n* are shown in Tables 3–5 (pages 324-326).

Positive animals may be either those that die or those that survive, depending on the type of test, as follows :

1. *Animals that die are counted as positive in the case of :*

(*a*) titration of live virus vaccine (chapters 19, 26, 27, 34) ;

(*b*) titration of challenge virus in the NIH test (chapter 33) ;

(*c*) titration of challenge virus in the quantitative assay and potency test of antirabies serum (chapter 40) ;

(*d*) the virus neutralization index test (chapter 8) ; and

(*e*) the Habel test and the modified Habel test for potency (chapters 31, 32).

2. *Animals that survive are counted as positive in the case of :*

(*a*) titration of sera by the "serum dilution—constant virus" technique (chapter 40) ;

(*b*) determination of the antigenic value of a vaccine by the "vaccine dilution—constant virus" technique (NIH test, chapter 40).

Calculation of the 50% endpoint dilution comprises the following steps :

Step 1 : Note the $\log_{10}$ of the reciprocal of the lowest dilution (of virus, serum, or vaccine) at which all the animals are positive (i.e., if the dilution is 10^{-3}, take $\log_{10} 10^3 = 3$).

Step 2. Determine the total number of positive animals at the dilution defined in step 1 and at all higher dilutions.

Step 3. Read from the table for the appropriate dilution factor the numerical value attributed to the number of positive animals determined in step 2.

Step 4. Add up the values determined in steps 1 and 3. This total represents the $\log_{10}$ of the reciprocal of the 50% endpoint dilution.

TABLE 1. EXAMPLES OF TITRATION OF VIRUS SUSPENSIONS (POSITIVE ANIMALS ARE THOSE THAT DIE)

	Example 1 $n = 5$		Example 2 $n = 5$		Example 3 $n = 5$		Example 4 $n = 10$		Example 5 $n = 10$		Example 6 $n = 4$	
Dilution (dilution factor = 10):	*pos.*	*neg.*	*pos.*	*neg.*	*pos.*	*neg.*	*pos.*	*neg.*	*pos.*	*neg.*	*pos.*	*neg.*
10^{-1}	—	—	5	0	—	—	6	4	—	—	4	0
10^{-2}	—	—	4	1	—	—	5	5	—	—	3	1
10^{-3}	5	0	0	5	5	0	7	3	—	—	1	3
10^{-4}	4	1	—	—	5	0	2	8	—	—	0	4
10^{-5}	5	0	—	—	5	0	0	10	10	0	0	4
10^{-6}	2	3	—	—	0	5	—	—	6	4	—	—
10^{-7}	0	5	—	—	0	5	—	—	1	9	—	—
Calculation step:												
1	3.0		1.0		5.0		0.0[a]		5.0		1.0	
2	16		9		5		30[a]		17[b]		8	
3[c]	2.7		1.3		0.5		2.5		1.2		1.50	
4	5.7		2.3		5.5		2.5		6.2		2.50	
Endpoint dilution	$10^{-5.7}$		$10^{-2.3}$		$10^{-5.5}$		$10^{-2.5}$		$10^{-6.2}$		$10^{-2.5}$	

[a] The lowest dilution at which all animals are regularly positive is assumed to be $10^{0.0}$ (i.e., undiluted virus suspension). This may be justified in this example, since the range of virus dilutions with both positives and negatives usually covers 2–3 tenfold steps only.

[b] It is assumed that no positives would have occurred at higher dilutions than 10^{-7}, had they been tested

[c] See Table 5.

TABLE 2. EXAMPLES OF TITRATION OF SERA AND VACCINES (POSITIVE ANIMALS ARE THOSE THAT SURVIVE)

Serum						Vaccine		
Example 7 $n = 5$ *(dilution factor = 2)*			Example 8 $n = 6$ *(dilution factor = 5)*			Example 9 $n = 16$ *(dilution factor = 5)*		
Dilution	*pos.*	*neg.*	*Dilution*	*pos.*	*neg.*	*Dilution*	*pos.*	*neg.*
$10^{-2.7}$	5	0	$10^{-0.7}$	6	0	$10^{-0.7}$	10	6
$10^{-3.0}$	4	1	$10^{-1.4}$	5	1	$10^{-1.4}$	8	8
$10^{-3.3}$	1	4	$10^{-2.1}$	4	2	$10^{-2.1}$	1	15
$10^{-3.6}$	1	4	$10^{-2.8}$	0	6			
$10^{-3.9}$	0	5						
Calculation step:			*Calculation step:*			*Calculation step:*		
1	2.70		1	0.70		1	0.0[a]	
2	11		2	15		2	35	
3[b]	0.51		3[c]	1.40		3[c]	1.18	
4	3.21		4	2.10		4	1.18	
Endpoint dilution	$10^{-3.21}$		*Endpoint dilution*	$10^{-2.10}$		*Endpoint dilution*	$10^{-1.18}$[a]	

[a] It is by no means certain that 16 out of 16 animals would have survived — as is assumed here — when inoculated with the undiluted vaccine. The estimate of the endpoint dilution is therefore a very rough one and probably an overestimate.

DILUTION FACTOR = 2

Total no. of positive animals	No. of inoculated animals per dilution		
	$n = 4$	$n = 5$	$n = 6$
4	0.15		
5	0.23	0.15	
6	0.30	0.21	0.15
7	0.38	0.27	0.20
8	0.45	0.33	0.25
9	0.53	0.39	0.30
10	0.60	0.45	0.35
11	0.68	0.51	0.40
12	0.75	0.57	0.45
13	0.83	0.63	0.50
14	0.90	0.69	0.55
15	0.98	0.75	0.60
16	1.05	0.81	0.65
17	1.13	0.87	0.70
18	1.20	0.93	0.75
19	1.28	0.99	0.80
20	1.35	1.05	0.85
21	1.43	1.11	0.90
22	1.51	1.17	0.95
23	1.58	1.23	1.00
24	1.66	1.29	1.05
25	1.73	1.35	1.10
26	1.81	1.41	1.15
27	1.88	1.48	1.20
28	1.96	1.54	1.25
29	2.03	1.60	1.30
30	2.11	1.66	1.35
31	for each further positive animal add 0.075	1.72	1.40
32		1.78	1.45
33		1.84	1.51
34		1.90	1.56
35		1.96	1.61
36		2.02	1.66
37		2.08	1.71
38		2.14	1.76
39		2.20	1.81
40		2.26	1.86
		for each further positive animal add 0.060	for each further positive animal add 0.050

TABLE 4. NUMERICAL VALUES FOR CALCULATION OF TITRES: DILUTION FACTOR = 5

Total no. of positive animals	No. of inoculated animals per dilution			
	$n = 4$	$n = 5$	$n = 6$	$n = 16$
4	0.35			
5	0.52	0.35		
6	0.70	0.49	0.35	
7	0.87	0.63	0.47	
8	1.05	0.77	0.58	
9	1.22	0.91	0.70	
10	1.40	1.05	0.82	
11	1.57	1.19	0.93	
12	1.75	1.33	1.05	
13	1.92	1.47	1.16	
14	2.10	1.61	1.28	
15	2.27	1.75	1.40	
16	2.45	1.89	1.51	0.35
17	2.62	2.03	1.63	0.39
18	2.80	2.17	1.75	0.44
19	2.97	2.31	1.86	0.48
20	3.15	2.45	1.98	0.52
21	for each further positive animal add 0.175	2.59	2.10	0.57
22		2.73	2.21	0.61
23		2.87	2.33	0.66
24		3.01	2.45	0.70
25		3.15	2.56	0.74
26		3.29	2.68	0.79
27		3.42	2.80	0.83
28		3.56	2.91	0.87
29		3.70	3.03	0.92
30		3.84	3.15	0.96
31		for each further positive animal add 0.140	3.26	1.00
32			3.38	1.05
33			3.49	1.09
34			3.61	1.14
35			3.73	1.18
36			3.84	1.22
37			for each further positive animal add 0.1165	1.27
38				1.31
39				1.35
40				1.40
41				1.44
42				1.49
43				1.53
44				1.57
45				1.62
				for each further positive animal add 0.0437

DILUTION FACTOR = 10

Total no. of positive animals	No. of inoculated animals per dilution				
	n = 4	*n* = 5	*n* = 6	*n* = 8	*n* = 10
4	0.50				
5	0.75	0.5			
6	1.00	0.7	0.50		
7	1.25	0.9	0.67		
8	1.50	1.1	0.83	0.50	
9	1.75	1.3	1.00	0.63	
10	2.00	1.5	1.17	0.75	0.5
11	2.25	1.7	1.33	0.88	0.6
12	2.50	1.9	1.50	1.00	0.7
13	2.75	2.1	1.67	1.13	0.8
14	3.00	2.3	1.83	1.25	0.9
15	3.25	2.5	2.00	1.38	1.0
16	3.50	2.7	2.17	1.50	1.1
17	3.75	2.9	2.33	1.63	1.2
18	4.00	3.1	2.50	1.75	1.3
19	4.25	3.3	2.67	1.88	1.4
20	4.50	3.5	2.83	2.00	1.5
21	4.75	3.7	3.00	2.13	1.6
22	5.00	3.9	3.17	2.25	1.7
23	5.25	4.1	3.33	2.38	1.8
24	5.50	4.3	3.50	2.50	1.9
25	5.75	4.5	3.67	2.63	2.0
26	for each further positive animal add 0.25	4.7	3.83	2.75	2.1
27		4.9	4.00	2.88	2.2
28		5.1	4.17	3.00	2.3
29		5.3	4.33	3.13	2.4
30		5.5	4.50	3.25	2.5
31		for each further positive animal add 0.2	4.67	3.38	2.6
32			4.83	3.50	2.7
33			5.00	3.63	2.8
34			5.17	3.75	2.9
35			5.33	3.88	3.0
36			for each further positive animal add 0.167	4.00	3.1
37				4.13	3.2
38				4.25	3.3
39				4.38	3.4
40				4.50	3.5
41				for each further positive animal add 0.125	3.6
42					3.7
43					3.8
44					3.9
45					4.0
					for each further positive animal add 0.1

Procedure using the Spearman-Kärber Formula

Where the simplified procedure described above is inapplicable, e.g., because the value of n at one or more dilutions has been reduced by accidental deaths, it is necessary to calculate the endpoint dilution from the Spearman-Kärber formula :

$$\log_{10} \text{ endpoint dilution} = -\left(x_o - \frac{d}{2} + d\sum \frac{r_i}{n_i}\right)$$

where :

x_0 = $\log_{10}$ of the reciprocal of the lowest dilution at which all animals are positive

d = $\log_{10}$ of the dilution factor

n_i = number of animals used at each individual dilution (after discounting accidental deaths)

r_i = number of positive animals (out of n_i).

Summation is started at dilution x_o.

Example 10. Endpoint dilution of a virus suspension

dilution	$10^{-3.6}$	$10^{-4.6}$	$10^{-5.6}$	$10^{-6.6}$	$10^{-7.6}$
positives (r_i)	5	5	0	1	0
inoculated (n_i)	5	5	5	4	5

$x_o = 4.6$; $d = 1.0$

$$\begin{aligned}\log_{10} \text{ of endpoint dilution} &= -\left[4.6 - \frac{1.0}{2} + 1.0\left(\frac{5}{5} + \frac{0}{5} + \frac{1}{4} + \frac{0}{5}\right)\right] \\ &= -(4.6 - 0.5 + 1.25) \\ &= -5.35\end{aligned}$$

Endpoint dilution = $10^{-5.35}$

Example 11 : Endpoint dilution of vaccine, NIH-potency test

dilution	$10^{-0.7}$	$10^{-1.4}$	$10^{-2.1}$	$10^{-2.8}$
r_i	10	8	1	0
n_i	15	14	16	15

If it is assumed that at dilution $10^{0.0}$ 16 animals would have been inoculated and that all of them would have been positive, then $x_o = 0.0$ and $d = 0.7$, and

$\log_{10}$ of endpoint dilution =

$$-\left[0.0 - \frac{0.7}{2} + 0.7\times\left(\frac{16}{16} + \frac{10}{15} + \frac{8}{14} + \frac{1}{16} + \frac{0}{15}\right)\right]$$

assumption. It is equally possible that not all the animals inoculated with undiluted vaccine would have been positive, which would have given a lower endpoint dilution.

Let it be assumed that the test is repeated, but, starting with the undiluted vaccine, and that the results are as follows :

dilution	$10^{0.0}$	$10^{-0.7}$	$10^{-1.4}$	$10^{-2.1}$	$10^{-2.8}$
r_i	13	9	9	0	0
n_i	15	15	13	14	15

Even with undiluted vaccine not all the treated animals have been protected, but on the basis of these more complete results it seems reasonable to assume that the "next" dilution step to the left of $10^{0.0}$ (i.e., $10^{0.7}$, $x_0 = -0.7$) would have given 100 % protection. Consequently,

$\log_{10}$ of endpoint dilution =

$$-\left[-0.7-\frac{0.7}{2}+0.7\times\left(\frac{15}{15}+\frac{13}{15}+\frac{9}{15}+\frac{9}{13}+\frac{0}{14}\right)\right]$$
$$=-(-0.7-0.35+0.7\times 3.16)$$
$$=-1.16.$$

Special Considerations

When using the Spearman-Kärber method it has to be borne in mind that random variations in the numbers that die will cause small but unknown deviations from the true values of the endpoint dilutions. These deviations will be larger if only a small number of animals is used. There may also be slight inaccuracies resulting from the method of estimation itself, but it has been shown that, on average, such inaccuracies are smaller with the Spearman-Kärber method than with other comparable methods (e.g., using the Reed & Muench formula).

For a material with a symmetrical dose-response curve, the value calculated by the Spearman-Kärber formula is the dilution that would cause a positive response in exactly 50% of the animals in a very large population, i.e., the 50% endpoint dilution. Sera and vaccines usually have symmetrical dose-response curves. On the other hand, the dose-response curves of virus suspensions are known to exhibit a certain degree of asymmetry. The result of this is that the endpoint dilution of virus suspensions as calculated by the Spearman-Kärber method is the dilution that would cause the death of exactly 43% of the animals, i.e., the 43% endpoint dilution. However, this value is just as suitable for measuring the infectivity of a virus suspension as is the 50% endpoint dilution. In this chapter, the term "50% endpoint dilution" or simply "endpoint dilution" refers to the true 50% endpoint dilution in the case of sera and vaccines and to the

43% endpoint dilution in the case of virus suspensions. To avoid confusion, some authors prefer to use the term "mean effective dose" in this sense.

For the Spearman-Kärber formula to be applicable it is necessary to use a range of dilutions wide enough to include both the dilution at and below which 100% of the animals will regularly be positive and the dilution at and above which 100% of the animals will regularly be negative. If one or both of these conditions is not met, it is sometimes assumed that, for a constant dilution factor, the next higher or lower dilution to the last one tested would have produced the desired result. The "fabrication" of data in this way is without any theoretical basis, but if applied with suitable caution it may do little harm (see footnotes to examples 4, 5, and 9, Tables 1 and 2). However, it is preferable to repeat the titration with a more appropriate range of dilutions, and this is essential if there are serious shortcomings in the data (see example 11). As already mentioned, the simplified procedure can be used only when the number (n) of mice per dilution is constant: any accidental death reduces n for the dilution concerned, and the longer procedure described on pages 327 & 328 must then be applied. However, a single accidental death may be disregarded if it occurs in a dilution at least 10 times lower than the lowest dilution at which all the inoculated animals are positive, or in a dilution at least 10 times higher than the highest dilution at which all the inoculated animals are negative. In the following example, therefore, where $n = 6$, the accidental deaths at dilutions 10^{-1} and 10^{-6} may be disregarded.

virus dilution:	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
positive:	5	6	4	1	0	0
negative:	0	0	2	5	6	5

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B. METHOD OF REED & MUENCH

Although there are reasons for preferring the Spearman-Kärber method of calculation, the method of Reed & Muench is still in use in some laboratories. The prerequisites are the same as for the Spearman-Kärber method, viz., constant number of animals per dilution, a constant dilution deaths do occur, preference should be given to the Spearman-Kärber formula (see Examples 10 and 11, page 327).

The starting point for the calculation of 50% endpoint dilutions (LD_{50} titres) by the Reed & Muench method is the dilution showing a mortality next below 50% ("starting point dilution"). The formula given below is used to determine the difference between the logarithm of the starting point dilution and the logarithm of the 50% endpoint dilution ("difference of logarithms"). If the mortality decreases with increasing dilution (as in titrations of virus suspensions) the 50% endpoint dilution will be lower than the starting point dilution. The "difference of logarithms" has therefore to be substracted from the logarithm of the reciprocal of the starting point dilution. On the other hand, the "difference of logarithm" has to be added if the mortality increases with increasing dilution. This distinction, which is illustrated in the following examples, must always be borne in mind when making calculations.

Example 1. Titration of virus suspension

Suppose that in a Habel test for potency the titration of the virus suspension in control mice gives the following results :

Serum dilution	*Mice*		*Cumulative totals*		*Percentage mortality*
	Survived	*Died*	*Survived*	*Died*	
10^{-5}	0	10	0	↑ 17	17/17 = 100
10^{-6}	4	6	4	7	7/11 = 64
10^{-7}	9	1	↓ 13	1	1/14 = 7

Totals are accumulated from 10^{-5} to 10^{-7} for survivors and from 10^{-7} to 10^{-5} for mice considered as having died of rabies.

In this example the dilution factor is 10 and the starting point dilution (showing a mortality next below 50%) is 10^{-7}.

Calculate the "difference of logarithms" from the formula :

$$\frac{50\% - (\text{mortality next below } 50\%)}{(\text{mortality next above } 50\% - (\text{mortality next below } 50\%)} \times \text{logarithm of dilution factor}$$

Hence,

$$\text{"difference of logarithms"} = \frac{50-7}{64-7} \times 1$$

$$= \frac{43}{57} \times 1$$

$$= 0.75$$

Since, in this example, the mortality decreases with increasing dilution, the 50% endpoint dilution is lower than the starting point dilution and is calculated by subtracting the "difference of logarithms" as follows :

log (reciprocal of 50% endpoint dilution) = log (reciprocal of starting point dilution)— "difference of logarithms"
= log $10^7 - 0.75$
= $7 - 0.75$
= 6.25

Hence,
log (50% endpoint dilution) = -6.25
and 50% endpoint dilution (LD_{50} titre) = $10^{-6.25}$

Example 2. Titration of rabies vaccine

Suppose that in the NIH potency test the titration of a rabies vaccine gives the following results:

Dilution of 5% brain tissue vaccine	*Brain tissue (mg)*	*Number of mice*	*Survived*	*Died*	*Cumulative totals*		*Percentage mortality*
					Survived	*Died*	
5^{-1} (= $10^{-0.7}$)	10	16	10	6	↑ 19	6	6/25 = 24
5^{-2} (= $10^{-1.4}$)	2	16	8	8	9	14	14/23 = 61
5^{-3} (= $10^{-2.1}$)	0.4	16	1	15	1	↓ 29	29/30 = 97

In this example the dilution factor is 5 and the starting point dilution[1] (showing a mortality next below 50%) is 5^{-1}.

Using the same formula as in the previous example, the "difference of logarithms" is:

$$\frac{50-24}{61-24} \times 0.699 = 0.491$$

Since in this case, the mortality is increasing as the dilution increases, the 50% endpoint dilution will be higher than the starting point dilution and will be calculated by adding the "difference of logarithms" as follows:

log (reciprocal of 50% endpoint dilution) = log (reciprocal of starting point dilution) + "difference of logarithms"
= $0.699 + 0.491$
= 1.2 (approx.)

Hence,
log (50% endpoint dilution) = -1.2
and 50% endpoint dilution = $10^{-1.2}$ (= 1 : 16)

---

[1] In some textbooks the starting point dilution for titrations of vaccines and sera (increasing mortality with increasing dilutions) is taken as the dilution showing a mortality next above 50%. The formula for the "difference of logarithms" then becomes

$$\frac{\text{(mortality at dilution next above 50\%)} - 50\%}{\text{(mortality next above 50\%)} - \text{(mortality next below 50\%)}} \times \text{logarithm of dilution factor}$$

Suppose that a typical protocol of the titration of a therapeutic serum gives the following results :

Serum dilution	*Mice*		*Cumulative totals*		*Percentage mortality*
	Survived	*Died*	*Survived*	*Died*	
$10^{-2.7}$ (1 : 500)	5	0	↑ 11	0	0/11 = 0
10^{-3} (1 : 1000)	4	1	6	1	1/7 = 14
$10^{-3.3}$ (1 : 2000)	1	4	2	5	5/7 = 71
$10^{-3.6}$ (1 : 4000)	1	4	1	9	9/10 = 90
$10^{-3.9}$ (1 : 8000)	0	5	0	↓ 14	14/14 = 100

In this example the dilution factor is 2 and the starting point dilution (showing a mortality next below 50%) is 10^{-3}.

Using the same formula as in the previous example the "difference of logarithms" is :

$$\frac{50-14}{71-14}\times 0.301 = 0.19$$

Since in this case the mortality is increasing as the dilution increases, the 50% endpoint dilution will be higher than the starting point dilution and will be calculated by adding the "difference of logarithms" as follows :

log (reciprocal of 50% endpoint dilution) = log (reciprocal of starting point dilution) + "difference of logarithms"
= $3 + 0.19$
= 3.2 (approx.)

Hence, log 50% endpoint dilution = -3.2
and 50% endpoint dilution = $10^{-3.2}$

C. INFECTIVITY TITRATION BASED ON PLAQUE COUNTS

When plaque counts are made on several plates at each dilution of a series of dilutions of the virus suspension, the concentration of plaque-forming units (PFU) in the undiluted suspension may be calculated from the sum of all the counts taken together, provided that two requirements are fulfilled :

(1) The mean plaque number must be inversely proportional to the dilution. In other words, the mean plaque number at a given initial dilution should—approximately at least—be ten times the mean plaque number at a tenfold dilution. This relation is unlikely to hold if overcrowding effects are present on plates with large numbers of plaques. The plaque

numbers counted at those dilutions will then be smaller on the average than expected from the numbers at higher dilutions. In order to ensure correct counts, all dilutions that give rise to overlapping and unrecognizable plaques should be eliminated from the calculation.

(2) The variation between plaque counts on plates of the same dilution should not exceed that to be expected from random fluctuation alone. If two plates are used at each dilution, a simple calculation yields a statistical coefficient D, the magnitude of which indicates whether random fluctuation is sufficient to account for the difference between the two counts. Let n_1 and n_2 be the pair of plaque numbers observed at the same dilution, then

$$D = \frac{(n_1 - n_2)^2}{n_1 + n_2}$$

If D does not exceed 5.02, the difference between n_1 and n_2 can be taken as due to random fluctuation. A value of D larger than 5.02 may be an indication of other sources of variation, for example a lack of homogeneity in the monolayer preparations or technical errors in pipetting.

Example

Suppose that the titration of a virus suspension gives the following results :

Data for calculating PFU of undiluted virus suspension					Data for calculation o coefficient D		
Dilution[a] (d)	*Number of plates* (k)	*Number of plaques* *Plate 1* (n_1)	*Plate 2* (n_2)	*Sum of plaques* (n_1+n_2)	*Difference of plaques* (n_1-n_2)	$(n_1-n_2)^2$	$D=\frac{(n_1-n_2)^2}{n_1+n_2}$
10^{-5}	2	27	38	65	−11	121	1.862
10^{-6}	2	6	3	9	3	9	1.000
10^{-7}	2	0	0	0			
Total number of plaques (N) =				74			

[a] Plaque counts on plates inoculated with lower dilutions are unreliable owing to overlapping of plaques ; such plates are therefore excluded from the calculation.

In this example the coefficient D is ≤ 5.02 at each dilution. The variation in the plaque counts can therefore be taken to be due to random fluctuation alone.

The following formula is used for calculating the number of PFU per inoculum of the undiluted suspension :

$$x = \frac{N}{\Sigma k \cdot d}$$

where

N = total number of plaques counted on all plates used for the evaluation of x

d = dilution

$$\begin{aligned}\Sigma\, k\cdot d &= 2\times 10^{-5} + 2\times 10^{-6} + 2\times 10^{-7}\\ &= 2\times 10^{-7}\,(10^{2} + 10^{1} + 10^{0}) = 2\times 10^{-7}\times 111 = 222\times 10^{-7}\end{aligned}$$

Substituting this value in the formula given above :

$$x = \frac{74}{222\times 10^{-7}} = 0.3333\times 10^{7} = 3\,333\,000 \text{ PFU per inoculum.}$$

If 0.1 ml of inoculum was used per plate, this corresponds to 3.333×10^{6} PFU per 0.1 ml of the undiluted suspension (or 3.333×10^{7} PFU per 1 ml). It should be noted that in calculating $\Sigma\, k\cdot d$ those plates that give zero plaque counts are also included.

The calculated value of x can only be considered as an approximation to the true value of the concentration of PFU in the undiluted suspension. The accuracy of this approximation is expressed by the standard deviation $s(x)$, where

$$s(x) = \sqrt{\frac{x}{\Sigma\, k\cdot d}}$$

Hence, the true concentration of PFU in the original suspension may be expressed as

$$x \pm s(x)$$

In the above example

$$\begin{aligned}s(x) = \sqrt{\frac{0.3333\times 10^{7}}{222\times 10^{-7}}} &= \sqrt{0.0015\times 10^{14}} = 0.0388\times 10^{7}\\ &= 0.388\times 10^{6}\end{aligned}$$

Hence, the estimated number of PFU per 0.1 ml of undiluted suspension is:

$$\begin{aligned}x \pm s(x) &= (3.333 \pm 0.388)\times 10^{6}\\ &\text{or } (3.333 \pm 0.388)\times 10^{7} \text{ PFU per ml.}\end{aligned}$$

Another way of making a statement about the accuracy of x is to calculate the coefficient of variation (c.v.) :

$$\text{Coefficient of variation} = \frac{\text{standard deviation of PFU}}{\text{calculated number of PFU}}$$

$$\text{or c.v.}(x) = \frac{s(x)}{x}$$

This is sometimes expressed as a percentage. In the example above,

$$\text{c.v.}(x) = 0.388/3.333 = 0.116 \text{ or } 11.6\%$$

There is no rule that the number of plates must be the same at each dilution. Sometimes a plate has to be rejected for some reason and cannot be included in the counts. To illustrate this, assume that at dilution 10^{-5} in the example above, there is only one plate and that this has 27 plaques. In this case we have $k = 1$ for that dilution, and $N = 27 + 9 = 36$

Then,

$$\begin{aligned}\Sigma\, k\cdot d &= 1\times 10^{-5} + 2\times 10^{-6} + 2\times 10^{-7}\\ &= 10^{-7}\,(10^{2} + 2\times 10^{1} + 2\times 10^{0}) = 122\times 10^{-7}.\end{aligned}$$

$$x = \frac{36}{122\times 10^{-7}} = 0.2951\times 10^{7} = 2.951\times 10^{6}\ \text{PFU per 0.1 ml.}$$

$$\begin{aligned}s\,(x) &= \sqrt{\frac{0.2951\times 10^{7}}{122\times 10^{-7}}} = \sqrt{0.002419\times 10^{14}} = 0.0492\times 10^{7}\\ &= 0.492\times 10^{6}\end{aligned}$$

$$x \pm s(x) = (2.951 \pm 0.492)\times 10^{6}\ \text{PFU per 0.1 ml}$$

$$\text{c.v.}\,(x) = \frac{0.492}{2.951} = 0.167 \text{ or } 16.7\%$$

A RAPID METHOD FOR FLUORESCEIN LABELLING OF RABIES ANTIBODIES

L. SCHNEIDER [1]

Any antirabies serum of known FA-potency may be labelled. Excellent results are obtained with antirabies globulins of hamster origin, as described in chapter 6.

The following procedures for the precipitation of globulins, for fluorescein labelling, and for the removal of unreacted dye by means of Sephadex have been used successfully in many diagnostic laboratories. With these methods fluorescein-labelled conjugates may be prepared ready for use within 2–3 days.

Materials

Ammonium sulfate : Saturated solution of $(NH_4)_2SO_4$, in distilled water, pH 7.2.

0.01 M phosphate-buffered saline (PBS)

Stock solution I :

sodium hydrogen phosphate, dihydrate (Na_2HPO_4, $2H_2O$) .	10.0 g
potassium dihydrogen phosphate (KH_2PO_4)	1.0 g
distilled water	1000 ml

Stock solution II :

sodium chloride	8.5 g
distilled water	1000 ml

Mix 180 ml of stock solution I with 900 ml of stock solution II. The pH of the resulting buffer should be 7.4–7.6.

Carbonate-bicarbonate buffer, pH 9

sodium carbonate, anhydrous (Na_2CO_3)	0.6 g
sodium bicarbonate ($NaHCO_3$)	3.7 g
distilled water	100 ml

The salts are brought into solution in individual flasks ; the solutions are then mixed and made up to the final volume.

Fluorescein isothiocyanate : Any product of proven quality may be used, either as pure, crystalline powder, or adsorbed to a carrier, such as Celite or other diatomaceous earth.

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[1] Federal Research Institute for Animal Virus Diseases, Tübingen, Federal Republic of Germany.

Sephadex : For removal of ammonium sulfate or unreacted fluorescein, columns made from Sephadex G25 or G50, medium, may be used advantageously instead of time-consuming dialysis.

About 100 g of Sephadex are stirred into 500 ml of 0.01 M PBS contained in a 1-litre flask. The gel is allowed to swell for at least 2 hours and the supernatant then removed by suction. The gel is next thoroughly mixed with about 900 ml of 0.01 M PBS and allowed to settle for exactly 5 minutes. After that time the supernatant is removed by suction. The process is repeated several times until the supernatant fluid clears within 5 minutes. This procedure removes small particles of Sephadex that would otherwise reduce the flow rate of the Sephadex column. The final gel may be stored at room temperature for long periods of time.

Precipitation of globulins by ammonium sulfate

All reactions are carried out at room temperature without precooling of the reagents.

1. To each 1.5 ml of serum add 1.0 ml of saturated ammonium sulfate solution dropwise under constant stirring.
2. Spin immediately at 2000 *g* for 5 minutes in a conventional laboratory centrifuge.
3. Discard supernatant fluid.
4. Resuspend the sediment in distilled water or 0.01 M PBS until the final volume equals the volume of the original serum.
5. Repeat steps 1–4 until the supernatant fluid remains clear (at least 3 times).
6. Take up the final sediment in half the original serum volume.
7. Remove the ammonium sulfate from the globulins by dialysis (see chapter 6, Annex, page 83) or by Sephadex filtration. For each 10 ml of globulin solution use a column of Sephadex gel of 200 mm × 15 mm, equilibrated with 0.01 M PBS (see above). Collect when an opalescent fraction appears.
8. Record the volume and determine the total proteins by the biuret reaction.

Labelling of globulins

1. Globulins free from sulfate ions are diluted to a concentration of 1–2% of protein with carbonate-bicarbonate buffer, pH 9. If the protein content is 1% or less, a quantity of carbonate-bicarbonate buffer equal to 10% of the total volume is added.
2. For each mg of protein add 0.015–0.02 mg of fluorescein isothiocyanate.

Fluorescein not bound to protein is rapidly and conveniently separated from globulins by use of a Sephadex column. For each 10 ml of conjugated globulins a column of Sephadex gel of at least 150 mm × 15 mm is required.

1. Fix a glass column of appropriate length (approx. 300 mm × 15 mm) in an upright position.
2. Fill the column with Sephadex gel equilibrated with 0.01 M PBS (see above).
3. Allow the gel to settle for several hours until the gel bed is evenly packed.
4. Rinse the column with 0.01 M PBS, taking care that the Sephadex gel never becomes dry.
5. Before use drain the buffer until the level has reached the top of the gel bed.
6. Carefully layer fluorescein-labelled globulins on top of the Sephadex gel. Avoid stirring up the gel.
7. After the globulins have entered the gel, add 0.01 M PBS on top of the column. Exert enough pressure to ensure an even flow.
8. Fluorescein-labelled globulins separate into two visible bands of orange colour. The first fraction, which rapidly approaches the lower end of the column, represents the fluorescein-labelled globulins. The second, slower moving fraction is unreacted dye. Collect all the first fraction.
9. Stop collecting when the colour intensity of the eluting fraction weakens, dispense in 1-ml amounts, and store frozen or freeze-dried.
10. Columns may be re-used after the Sephadex has been freed from residual, unreacted dye.

Working dilution of conjugate

1. Prepare serial twofold dilutions of Sephadex-filtered conjugate in 5% normal mouse brain (NMB) suspended in 0.01 M PBS. For weak sera a dilution range of 1 : 5 to 1 : 40 is appropriate. For conjugated hamster globulins dilutions of 1 : 40 to 1 : 160 are indicated.
2. Stain smears containing rabies antigen as described in chapter 6. The highest dilution producing brilliant fluorescence is considered the working dilution of the conjugate.
3. The routine preparation and staining of test and control slides is carried out as described in chapter 6.

APPENDIX 3

CELL MONOLAYER PLAQUE TEST

L. SCHNEIDER [1]

The classical plaque test (Dulbecco, 1952) has not seemed to be useful for the plaquing of rabies virus (RV). This test involves infection of monolayer cell cultures, which are subsequently overlaid with a solid or semisolid layer of plaquing medium containing agar, agarose, or methylcellulose. It has recently been found that if the agar is replaced by Sephadex G200 a plaquing medium is obtained that permits the plaquing of RV in a cell monolayer (Schneider, unpublished data).

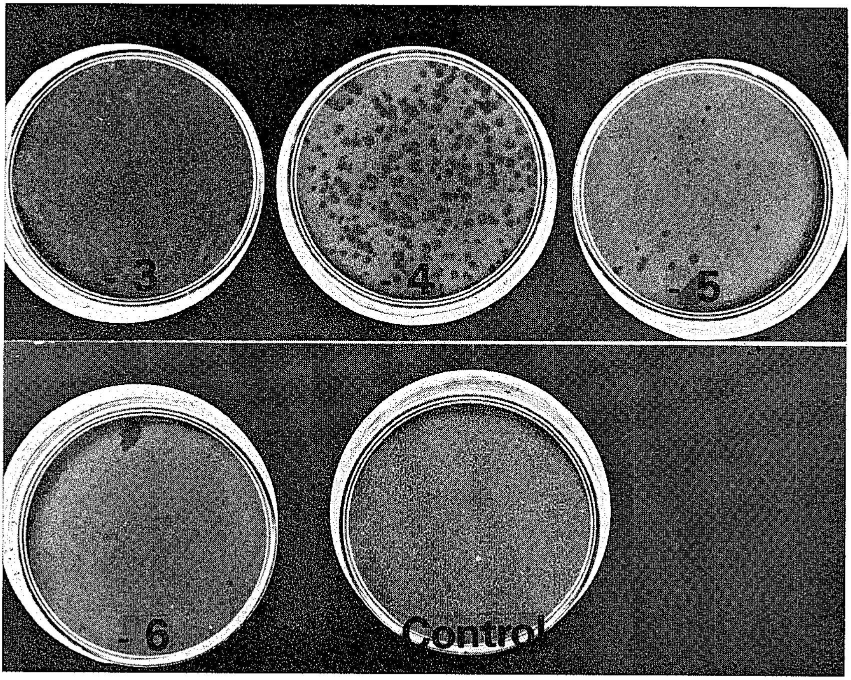
For the Sephadex monolayer plaque (SMP) test, Sephadex is suspended in the plaquing medium at a concentration that allows the gel beads to form a thin but complete layer on the cell sheet. Excess plaquing medium remains on top of the Sephadex layer in order to keep the cells viable. Since the Sephadex beads tend to move freely in the medium, the Petri dishes should not be disturbed during the period of incubation (Schwöbel, 1969).

In the buffer system Tris chloride has now been replaced by Tes (see below), making it possible to perform the SMP test in the absence of carbon dioxide (Richter, 1967).

So far, plaque formation has been achieved with several strains of RV in BHK-S13 cells (see figure) and in three other lines of BHK-21 cells. The number of plaques was equal in all cell systems, but larger plaques were observed in S13-cells. Plaque formation was suppressed by specific immune sera in all cell systems tested.

The Sephadex monolayer plaque test is a convenient method that can be handled by any laboratory personnel with some experience in tissue culture work. The ease of preparing, infecting, and staining monolayer cultures, the omission of carbon dioxide during incubation, and the good visibility of permanently stained or even of unstained plaques are definite advantages of this method.

The SMP technique may be used for infectivity titrations of tissue-culture-adapted strains of RV ; for the cloning of RV ; and for the plaque reduction test as described in chapter 9, page 113.



Materials

Growth medium

Growth medium for the propagation of cells consists of BHK-21 medium (see chapter 9, Annex 1), supplemented with tryptose phosphate (0.3% w/v), lactalbumin hydrolysate (0.5% w/v), normal calf serum (10% v/v), and antibiotics.

Plaquing medium

For 1 litre of medium the following ingredients are mixed :

distilled water	795 ml	
balanced salt solution (10-fold concentrate) [1]	100 ml	
amino acids (100-fold concentrate) [1]	10 ml	
vitamins (100-fold concentrate) [1]	10 ml	
glutamine (100-fold concentrate) [1]	10 ml	
Tes, 1 M [2]	27.5 ml	
penicillin	500 000 IU	
streptomycin	200 mg	(156 000 IU)
mycostatin	50 000 IU	
fetal calf serum	20 ml	

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[1] For formula see Chapter 9, Annex 1, page 120.

[2] 229.2 g of *N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid dissolved in 1000 ml of distilled water.

The pH is brought to 7.2 with 1 M sodium hydroxide solution ; about 25 ml are needed for each litre of medium. The pH of 7.2 was found to be optimum under conditions described here.

Sephadex G 200 [1] is filled as dry powder into flasks in portions of 20 g each, and is sterilized in a dry oven at 180°C for 1 hour.

To each litre of plaquing medium 20 g of sterilized Sephadex G 200 are added and allowed to swell for 24 hours at 4°C.

Virus diluent

The virus diluent has the same composition as the growth medium, except that the calf serum is replaced by bovine serum albumin (0.3% w/v), and DEAE-dextran is added to a final concentration of 50 μg/ml.

Cells

BHK-S13 cells are maintained and sub-cultivated as described in Chapter 9, page 103. Original BHK-21 cells as maintained for the propagation of RV may be used instead of S13 cells.

Plaquing procedures

Propagation of cells

1. Trypsinize confluent BHK monolayer cultures from 1-litre Roux flasks and suspend the cells in growth medium at a concentration of 10^6 cells per ml.

2. Dispense 5-ml portions of the diluted cell suspension into glass or plastic Petri dishes (60×15 mm). About 25 Petri dishes can be prepared from one Roux flask.

3. Incubate at 37°C in a moist incubator, at a carbon dioxide concentration of 3–5%. Confluent monolayers should form after 48 hours.

Virus infection

4. Remove the growth medium from the Petri dishes by suction and rinse the dishes twice with phosphate-buffered saline.

5. Infect the plates by placing 0.1 ml of virus inoculum on top of the cell sheet. Disperse by rotating the dishes. Infect at least 2 plates per virus dilution.

6. Allow the virus to adsorb to the cells by incubating the dishes at 34–37°C for 1 hour. Remove the residual inoculum by rinsing the plates with phosphate-buffered saline.

dish and incubate at 34–37°C in a moist incubator without carbon dioxide. Leave the plates undisturbed for 5–6 days.

Staining of plaques

8. Remove the plates from the incubator and fix the cell sheet by carefully pouring 5 ml of a 2–5% solution of formaldehyde in distilled water over the Sephadex layer. Fixation is complete after 30 minutes.

9. Rinse the plates under running tap water until the Sephadex grains are completely removed.

10. Add 3 ml of undiluted Giemsa stock solution. Stain for at least 20 minutes.

11. Remove the stain, rinse thoroughly with tap water, and allow the plates to dry in air.

12. Place the dried Petri dishes bottom up on top of a transparent light box. Count the number of plaques for each dish by marking each plaque with a wax pencil on the bottom of the plate.

13. Calculate the number of plaque-forming units (PFU/ml) using the example in Appendix 1, page 333. For the method of calculating the 50% plaque reduction endpoint see Chapter 9, page 114.

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APPENDIX 4

PROPOSED SAFETY TESTS FOR INACTIVATED RABIES VACCINE PREPARED IN CELL CULTURES

R. NETTER[1] *& F. T. PERKINS*[2]

A. VACCINES PREPARED IN HUMAN DIPLOID CELLS

The manufacturing requirements for new virus vaccines prepared in cell cultures are generally based on those for poliomyelitis vaccine. For killed vaccines some inactivating agents may permit the survival of live viruses and it is therefore necessary to exercise just as much vigilance with inactivated vaccines as with live vaccines.

Moreover, there is a well-founded fear that, when serious postvaccinal incidents occur, new viruses originating from cell cultures may be discovered even though they may have met the testing requirements. This uncertainty has led many investigators to turn to human diploid cells, demonstrated in advance to be free from viruses, for the preparation of vaccines (Hayflick & Moorhead, 1961). Requirements for the manufacture and testing of different live virus vaccines have been proposed at various scientific meetings, and requirements for oral poliomyelitis vaccine have been published by WHO.[3]

Inactivated rabies vaccine prepared in human diploid cells presents a problem of a general nature inasmuch as there are as yet no requirements for other inactivated vaccines prepared in human diploid cells. Nevertheless, the requirements that apply to the cell substrate must be similar whether the final vaccine is a killed or live attenuated virus suspension. In addition to this there are a number of specific problems that are summarized below:

(1) The Pasteur strain of fixed rabies virus or one of its derivatives should be used for vaccine production. Such strains are characterized by a short fixed incubation period after intracerebral inoculation into animals. On the other hand, with Fermi type vaccine, the pathogenicity after parenteral inoculation was reduced to such an extent that it was possible to tolerate the presence of living virus up to $10^{2.7}$ LD_{50} per 0.03 ml.[4] In high doses,

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[3] *Wld Hlth Org. techn. Rep. Ser.*, 1972, No. 486, Annex 1.

[4] *Wld Hlth Org. techn. Rep. Ser.*, 1973, No. 523 (Sixth report of the WHO Expert Com-

man; the maximum safety precautions must, therefore, be observed by those handling it. The approved strain must never have been passaged in an animal other than the rabbit, or in monkey kidney cells, or in a cell line. It is desirable that it should have been adapted directly on to human diploid cells.

(2) Only human diploid cells that have satisfied all the criteria of acceptability[1] and have been approved by the national authorities may be used. Their purity safeguards the purity of the vaccine.

(3) The techniques for rabies virus multiplication hitherto used have not enabled very high virus titres to be obtained. This has made it necessary to concentrate the product, giving relatively small vaccine lots.

(4) The vaccine must be inactivated by an approved method.

Seed Lots

Control of diploid cells

In general the controls on the cell substrate used for the production of the seed lots shall follow those applied to the same substrate used for the production of poliomyelitis vaccine (oral).[1]

1. *Control cells*

10% of the cells from the passage preceding the harvested passage are set aside as uninoculated controls and used for the following tests:

(*a*) *Titration of Echo virus or Sindbis virus.* In some countries groups of 5 tubes are seeded with each successive dilution of the virus. After incubation for 1 week at 37°C the titre expressed in $TCID_{50}$ should be within the normal range. If such a test is applied it is the responsibility of the National Control Laboratory to supply the reference virus suspensions.

(*b*) *Karyotype studies.* 300 cells in metaphase are examined for polyploidy, exact counts are made on 100 cells in metaphase, and a complete karyotype analysis is performed on a single cell. The permissible upper limit for the frequency of cells with chromosome breaks is 9%; the limit for structural abnormalities is 4%, that for polyploidy 5%.[1]

(*c*) *The absence of heterotransplantability.* Tests are made to show the freedom from heterotransplantability. One such test is by inoculating one million cells into each cheek pouch of 6 three-week-old hamsters, 3 of which have been treated with cortisone. The animals are observed for 3 months and their sensitivity is tested against a parallel series inoculated with an equivalent number of HeLa, KB or HEP (Hayflick & Moorhead, 1961) cells. Samples for histological examination are taken at the end of the procedure. Other tests of at least an equivalent sensitivity may also be used ; it has recently been found that tests in mice and hamsters under immune suppression by drugs or antilymphocyte serum give good results.

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1972, No. 486, see also Gušić, 1970.

(*d*) *The identity of the cells* with the approved cells is established by testing for antigens of the HL-A system, using the cytotoxicity or the complement fixation test (Lapeyre et al., 1970 ; Van Lan Dao, 1971–72) or by any other test of equivalent sensitivity.

(*e*) *Test for haemadsorbing viruses and examination of the remaining cells*. At the time of inoculating cultures with the seed virus, a haemadsorption test is carried out on 4% of the control cells, using human, guineapig, monkey, and chicken red cells. The test is repeated after observing the cells for 14 days. The results are read (i) after incubation for 30 minutes at 2–10°C, (ii) after another 30 minutes at ambient temperature, and (iii) after 30 more minutes at 37°C. No haemadsorption should be present.

(*f*) *Tests in animals for extraneous agents*. One million cells are inoculated intramuscularly into each of 10 mice less than 1 day old, from 2 different litters and a further 1 million cells into each of 10 adult mice weighing 18–20 g ; 2 million cells are inoculated intramuscularly into each of 5 guineapigs weighing about 350 g and a further 2 million cells into each of 5 rabbits weighing about 1.5 kg.

After 30 days at least 80% of the animals must remain in good health and death or disease in the remaining animals must not be attributable to the cells.

(*g*) *Test in embryonated eggs for extraneous agents*. One million cells are inoculated into the allantoic cavity of each of 10 embryonated eggs 9–11 days old. After 3 days the allantoic fluids should be free from haemagglutinins for chicken and guineapig red cells when incubated both at 2–10°C and at ambient temperature.

(*h*) *Tests in cell cultures for extraneous agents*. At the time of virus harvest from the production bottles, supernatant fluids shall be taken from the uninoculated control cultures and tested for the presence of extraneous agents as outlined in 2 (*c*) below.

2. *Cells used for the production of rabies seed virus*

(*a*) *Direct observation :* The cells should be of normal morphology at the time of inoculation with the virus.

(*b*) *Sterility tests :* 30 ml of pooled supernatant fluids obtained just before inoculation with the virus are distributed in media that permit the inoculation with the seed virus, samples of the supernatant fluids are taken and 10 ml are inoculated into each of the following cultures :

human embryo kidney cells ;

kidney cells of the *Cercopithecus* or *Erythrocebus* monkey ;

primary rabbit kidney cells.

The dilution of the inoculum in the nutrient medium should not exceed 1 in 4 and the area of the cell sheet shall be at least 3 cm^2 per ml of pooled fluid. The morphology of the cells must remain unchanged for 14 to 21 days. At the end of the observation period a subculture shall be made from the *Cercopithecus* and *Erythrocebus* cultures in the same cell system and these cultures shall be examined for a further 14 days. Staining tests on the last day will demonstrate the absence of specific inclusions. Once a week tests for haemadsorbing viruses are carried out on some of the cultures at 2–10°C, at ambient temperature, and at 37°C, using human, guineapig, monkey, and chicken red cells.

Control of the virus suspension before clarification

If it is not possible to carry out the following tests at the time of harvesting it will be necessary to freeze the samples at a temperature of −60°C or below. Samples so treated should not be frozen and thawed more than twice.

1. *Infective titre*

Mice weighing 15–20 g are used. Inoculate successive dilutions of the suspension intracerebrally (0.03 ml) into groups of at least 5 mice and intraperitoneally (0.1 ml) into other groups. At the end of the 15-day observation period the LD_{50} of the mice inoculated intracerebrally must be at least 1000 times that of the mice inoculated intraperitoneally.

2. *Purity and identity tests*

Examination for certain extraneous agents is performed by mixing 9 volumes of the virus suspension with 1 volume of antirabies serum having a sufficiently high titre to neutralize the whole of the virus suspension and being of neither human nor simian origin. The mixture is incubated for one-and-a-half hours at 37°C.

(*a*) *Tests in animals*

Mice weighing 12–15 g. Each of 20 mice is given 0.25 ml of the neutralized virus suspension by the intraperitoneal route, 0.02 ml by the intracerebral route, and 0.01 ml into the footpad. They are kept under observation for 28 days, and the animals must remain in good health. The cause of death must be established for any mice that die.

Suckling mice. Ten mice less than one day old or 20 mice less than one week old are given 0.1 ml of the neutralized suspension by the intraperitoneal route and 0.01 ml by the intracerebral route. They are kept under observation for 21 days and those dying or becoming ill from the second day onward must be examined to determine whether the virus suspension was responsible.

Guineapigs. 5 ml of the neutralized suspension are injected intraperitoneally into each of 5 guineapigs weighing 350–500 g. The animals are observed for 6 weeks ; at least 4 must survive and none of the animals must show evidence of infection with *Mycobacterium tuberculosis* or other disease attributable to the virus suspension.

Rabbits. Each of 5 rabbits weighing 1.5–2 kg is inoculated intracerebrally with 0.25 ml, subcutaneously with 2 ml, and intradermally with 1 ml of the neutralized suspension (0.1 ml at each of 10 points). They are kept under observation for 21 days. At least 4 rabbits must survive and none of them must show evidence of disease attributable to the virus suspension.

(*b*) *Tests in cell cultures*

Kidney cells of Cercopithecus *or* Erythrocebus *monkeys.* 10 ml of the neutralized virus suspension are seeded in culture bottles in such a manner that the dilution does not exceed 1 in 4 ; after 7 days a subculture of 10 ml of the pooled supernatant fluids is passaged on other monkey kidney cells. After the cultures have been kept under observation for 14 days at 37°C the following procedures are carried out :

(i) a test for haemadsorbing viruses, using guineapig red cells ;

(ii) intracerebral inoculation of 0.03 ml of supernatant into 5 mice weighing 12–15 g, followed by observation for 15 days ;

(iii) titration of a known virus by comparison with control cells in order to establish the absence of interference.

Human diploid cells. 10 ml of the neutralized virus suspension are seeded in culture bottles in such a manner that the dilution does not exceed 1 in 4 ; after 7 days a subculture of 10 ml of the pooled supernatant fluids is passaged on other human diploid cells. After the cultures have been kept under observation for 14 days at 37°C the following procedures are carried out :

(i) a test for haemadsorbing viruses, using guineapig red cells ;

(ii) intracerebral inoculation of 0.03 ml of the supernatant into 5 mice weighing 12–15 g, followed by observation for 15 days ;

(iii) titration of a known virus by comparison with a control series in order to establish the absence of interference.

Primary rabbit kidney cells. 10 ml of the neutralized virus suspension fluids is seeded on other primary rabbit kidney cells. After the cultures have been kept under observation for 14 days at 37°C the following procedures are carried out :

(i) a test for haemadsorbing viruses, using guineapig red cells ;

(ii) intracerebral inoculation of 0.03 ml of the supernatant into 5 mice weighing 12–15 g, followed by observation for 15 days ;

(iii) titration of a known virus by comparison with a control series in order to establish the absence of interference.

3. *Sterility tests*

30 ml of the non-neutralized virus suspension are distributed in media that permit the growth of aerobic and anaerobic organisms, of fungi, and of mycoplasma (see Chapter 24, page 226).

Production Lots

Uninoculated control cells

The cells used for the production of vaccine must be derived from the approved cell bank shown to be free from extraneous agents and heterotransplantability. The production of each batch of vaccine, however, uses a cell substrate that has been passaged many times from the passage level at which the cell bank was established. It is important, therefore, to establish that there has been no contamination of the particular cell substrate used for the production of each individual batch of vaccine.

Tests on control cells

The tests applied to the 10% uninoculated control cells set aside from each batch of cells used for vaccine production are identical to those applied to the control cells used in the production of the seed lot.

These are :

(*a*) Karyotype studies (see 1 (*b*), p. 344)
(*b*) Absence of heterotransplantability (see 1 (*c*), p. 344)
(*c*) Tests for the presence of haemadsorbing viruses (see 1 (*e*), p. 345)
(*d*) Test in animals for extraneous agents (see 1 (*f*), p. 345)
(*e*) Tests in embryonated eggs for extraneous agents (see 1 (*g*), p. 345)
(*f*) Tests in cell cultures for extraneous agents (see 1 (*h*), p. 345)

Cells used in vaccine production

Replace the growth medium by a maintenance medium containing no proteins of animal origin, no serum, and no sensitizing antibiotics, such as penicillin and streptomycin.

Tests on virus suspension before clarification

(*a*) *Mice weighing 15–20 g.* Comparative titration by the intracerebral and intraperitoneal routes (see p. 346).

(*b*) *Bacterial sterility test* (see p. 345)

20 ml are distributed in media that permit the growth of aerobic and anaerobic organisms, *Mycobacterium tuberculosis*, fungi, and mycoplasma.

Control of the virus suspension following concentration and inactivation

The virus shall be concentrated by a process approved by the national control authority. The inactivation curve shall be determined at appropriate intervals by titration of the virus in mice weighing 12–15 g. The total inactivation time should be at least double the time necessary to obtain viral sterility.

Tests in cell culture

(*a*) *Human diploid cells.* 25 ml of the inactivated concentrated suspension are inoculated into the maintenance medium for human diploid cells in such a manner that the dilution does not exceed 1 in 4 and the area of the cell sheet shall be at least 3 cm^2 per ml of pooled fluid. The cells are observed for at least 21 days. After 14 and 21 days samples of supernatant are taken for intracerebral inoculation of 0.03 ml into 10 mice weighing 12–15 g; the mice are observed for 21 days. At the end of the period of observation of the cells, the absence of cytopathogenicity is verified and a test for haemadsorbing viruses is carried out at 2–10°C and at ambient temperature, using guineapig red cells.

(*b*) *Kidney cells of* Cercopithecus *or* Erythrocebus *monkeys and primary rabbit kidney cells.* 10 ml of the inactivated concentrated suspension are inoculated into each of the kidney cell cultures from *Cercopithecus* and *Erythrocebus* monkeys in such a manner that the dilution in the maintenance medium does not exceed 1 in 4. The cells are observed for 14 days and at the end of this period tests are made to establish the absence of cytopathogenicity and the absence of haemadsorbing viruses, using guineapig red cells at 2–10°C and at ambient temperature.

Preparation and control of final bulk

1. *Preservatives and other substances added*

Any preservative added to the final bulk and its concentration shall be approved by the national control authority. No antibiotics shall be

Each final bulk shall be tested for sterility according to the requirements given in Part A section 6 of the Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).[1]

Control tests on final product

1. *Identity test*

An identity test shall be performed on at least one labelled container from each filling lot by an appropriate method. The test for immunogenicity may serve as an identity test.

2. *Sterility*

Each filling lot shall be tested for sterility (see above).

3. *Innocuity test*

Each filling lot shall be tested for abnormal toxicity by appropriate tests in mice and guineapigs using parenteral injections. The test procedures shall be approved by the national control authority. The following procedures are suggested :

(*a*) *Mice.* 10 mice weighing 12–15 g, originating from a stock guaranteed to be free from pathogenic organisms, and particularly from the virus of lymphocytic choriomeningitis and from polyoma virus, are inoculated as follows : 0.02 ml intracerebrally ; 0.25 ml intraperitoneally ; and 0.01 ml into the footpad. The mice are observed for 15 days and any death or suspected disease occurring from the second day onward must be investigated to show that the vaccine was not responsible.

(*b*) *Guineapigs (or rabbits).* Two guineapigs weighing 300–350 g or 2 rabbits weighing 1.25–1.5 kg are each inoculated intramuscularly with half a human dose. They are observed for 15 days during which time there should be no loss of weight.

4. *Other requirements*

The potency tests to be used are described in chapter 33. The vaccine should fulfil the requirements for freeze-dried vaccine after storage of samples for 1 month at 37°C (accelerated degradation test). Some countries stipulate that each filling lot shall be tested for residual moisture.

The requirements concerning inspection of final containers, records, labelling, distribution and shipping, and storage and expiry date shall comply with the appropriate requirements given in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories).[1]

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1960, No. 200.

B. VACCINES PREPARED IN PRIMARY CELL CULTURES OF ANIMAL ORIGIN

Many attempts are being made to prepare rabies vaccine in non-neuronal tissue by using tissue cultures. Calf kidney, hamster kidney, and chick embryo fibroblast cell cultures are already being investigated and some of the vaccines have already been licensed in different countries. With the introduction of such vaccines it would be desirable to establish requirements for their safety, taking into account all the techniques in virology that have been developed over the last decade.

Although it is not possible to give here specific recommendations about individual requirements, there are general principles that should be followed. These are :

1. The seed virus

All production should be based on a seed virus system using a virus strain shown to be stable and immunogenic in man. The virus seed must be shown to be free from extraneous agents.

2. Source of tissue for cell cultures

The tissue used for the preparation of cell cultures must be obtained from animals or birds known to be in good health and to be free from pathogenic diseases. The specification for the freedom from such diseases must be considered separately for each individual animal species.

3. Tests on the cell substrate

When primary tissue is used, each batch of cell cultures derived from an individual animal or pool of tissue represents a unique situation with respect to inherent contamination with extraneous agents. Over the last 14 years (i.e., since the first international requirements for inactivated poliomyelitis vaccine[2] were formulated), there have been several changes in the philosophy of testing virus vaccines for the presence of contaminants. It has been recognized that, in the presence of a large number of vaccine virus particles, it is extremely difficult to detect a small quantity of an extraneous agent that may be an inherent contaminant of the cell substrate.

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the tests to ensure that a contaminant has not been introduced into the virus harvest during production are made on 25% of each batch of cells set aside as uninoculated control tissue. This principle was adopted in the international requirements for inactivated measles vaccine,[1] and it is almost certainly the principle upon which all future requirements for inactivated virus vaccines will be based.

The tests on the 25% control cells involve the subculture of the supernatant fluids in several cell systems, as well as the examination of the control cells for the presence of haemadsorbing viruses by the addition of red blood cells of animal and avian origin.

4. Tests on the virus harvest

Having ensured that no extraneous agent arising from the cell substrate has contaminated the virus harvest, the only tests deemed necessary on the living virus harvest are the inoculation of media for the detection of bacteria (especially *Mycobacterium tuberculosis*), fungi, and mycoplasma.

5. Tests of inactivation

Great care should be taken to ensure that the inactivation procedure applied to the clarified virus harvests gives reproducible results. It is essential to carry out many tests for residual live virus on several production lots, as well as to ensure that the inactivated virus pool retains antigenicity. Precise details of the inactivation procedure should be recorded and adhered to in all future production lots.

6. Tests on inactivated final bulk

The tests for residual live virus are the same as those for any extraneous agent that may have survived the inactivation process. A realistic quantity of final bulk should be tested in several cell cultures, which should always include :

(*a*) the same cell culture system, but not the same cell batch, in which the virus was grown ;

(*b*) a human cell culture system ; and

(*c*) a monkey cell culture system.

The final bulk should also be tested in suckling and adult animals or in fertile eggs, depending on the contaminants that might be present in the cell substrate.

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1966, No. 329.

7. Tests on final product

All vaccine in the final ampoules must comply with the tests now established for other virus vaccines. These are tests for:

(*a*) identity;

(*b*) potency;

(*c*) innocuity;

(*d*) freedom from bacteria, fungi, and mycoplasma.

In addition, the final product must be shown to be stable and the storage conditions should be formulated accordingly.

Special conditions of labelling must be considered individually for each product.

Suggested procedures for many of the above tests are given in section A (page 350).

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A RAPID TISSUE CULTURE TEST FOR DETERMINING RABIES NEUTRALIZING ANTIBODY

J. S. SMITH,[1] *P. A. YAGER*[1] *& G. M. BAER*[1]

The *in vivo* (mouse) test developed over 30 years ago (Webster & Dawson, 1935) has been the most commonly used procedure for determining the level of rabies virus neutralizing (VN) antibody. In recent years, however, several laboratories have developed *in vitro* techniques for rabies VN antibody testing. In the USA, the California State Department of Health currently uses a fluorescent focus inhibition test (FFIT) in which LEP Flury rabies virus is mixed with test serum, and this combination is then added to a BHK cell monolayer, the resultant presence or absence of viral invasion being examined 4 days later by fluorescent antibody (FA) staining (Lennette & Emmons, 1971). Similar tests using CVS fixed virus on chick fibroblast cells (King et al., 1965) and ERA virus on BHK-21 cells (Debbie et al., 1972) have been described. The plaque neutralization technique using BHK-21/S13 cells suspended in agarose (Sedwick & Wiktor, 1967) has been shown to give good correlation with the mouse neutralization test (see chapter 8, page 94). We have recently developed a rapid FFIT (RFFIT) that can be performed in only 24 hours, and the results show close correlation with those of the mouse VN test in measuring VN antibody levels. In the RFFIT, BHK-21/S13 cells treated with DEAE-dextran are added to a mixture of test serum and tissue-culture-adapted rabies virus (incubated for 1 ½ hours) and monolayers are allowed to form. After 24 hours' incubation, the cell monolayer is examined by FA staining for the presence of non-neutralized rabies virus. The test is performed in the following manner :

Preparation of virus challenge

The rabies challenge virus is the CVS-11 strain (Kissling, 1958), propagated in BHK-21/S13 cells. The optimal challenge dose is the highest dilution of virus that gives strong FA staining in these cells after 24 hours ; [2] we have found that this dose is 0.16 PFU/cell or 16 000 PFU/0.1 ml of virus suspension.

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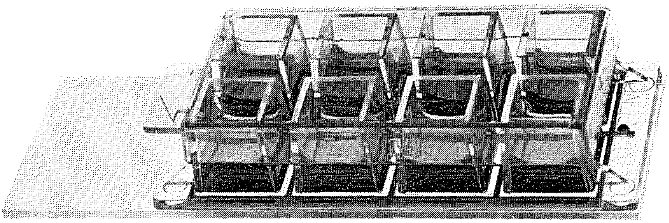
[2] If titrated against the NIH reference serum, this dilution should give VN antibody titres comparable to those obtained in the mouse VN index test.

Serum-virus mixture

All sera to be tested are inactivated at 56°C for 30 minutes. Sera frozen and held for longer than 2 months are reinactivated prior to testing. Animal sera must be treated with kaolin to adsorb non-specific inhibitors (Hierholzer et al., 1969) : an equal volume of 25% kaolin is added to each serum, and the mixture is allowed to stand at room temperature for 20 minutes with frequent agitation ; it is then centrifuged at 800 *g* for 10 minutes at room temperature. The supernatant liquid is considered to be a 1:2 dilution of the original serum. NIH reference serum is used as a control. Human sera may be tested at 1:5 and 1:50 dilutions (screening dilutions); for more exact quantification of antibody titres, fivefold serum dilutions may be made. Dilutions are made in Lab-Tek TC Chamber Slides[1] (Fig. 1) ; 0.1 ml of the proper dilution of challenge virus is added to 0.1-ml final volumes of all serum dilutions. The chambers are then incubated at 35°C in a controlled humidity carbon dioxide chamber for 90 minutes.

FIG. 1 : LAB-TEK TISSUE CULTURE CHAMBER SLIDES



Addition of cells

BHK-21/S13 cultures may be grown in plastic or glass containers. The growth medium is Eagle's MEM, as modified by MacPherson & Stoker (see chapter 9, Annex 1, page 120), and supplemented with 10% of inactivated fetal calf serum and 10% of tryptose phosphate broth. BHK monolayers 1–4 days old (between the 25th and 50th passage level) are trypsinized for use in the RFFIT. Immediately before their use in the RFFIT the cells are treated with a solution of DEAE-dextran[2] in growth medium (10 µg/ml) for 10 minutes at room temperature. Suspensions containing 1×10^5 cells/0.2 ml of growth medium are added to each of the 8 chambers of the Lab-Tek TC slides, and the slides are then returned to the carbon dioxide chamber.

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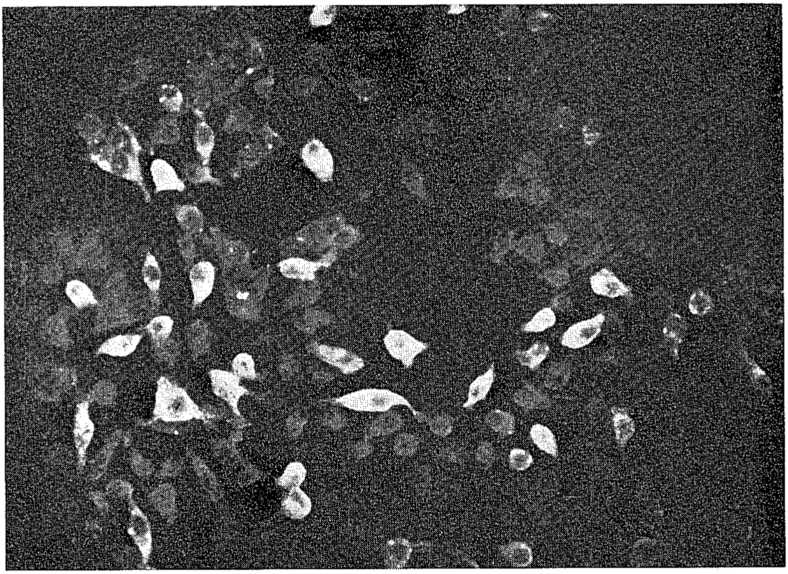
[1] Obtainable from Lab-Tek Products, Division of Miles Laboratories, Inc., Westmont,



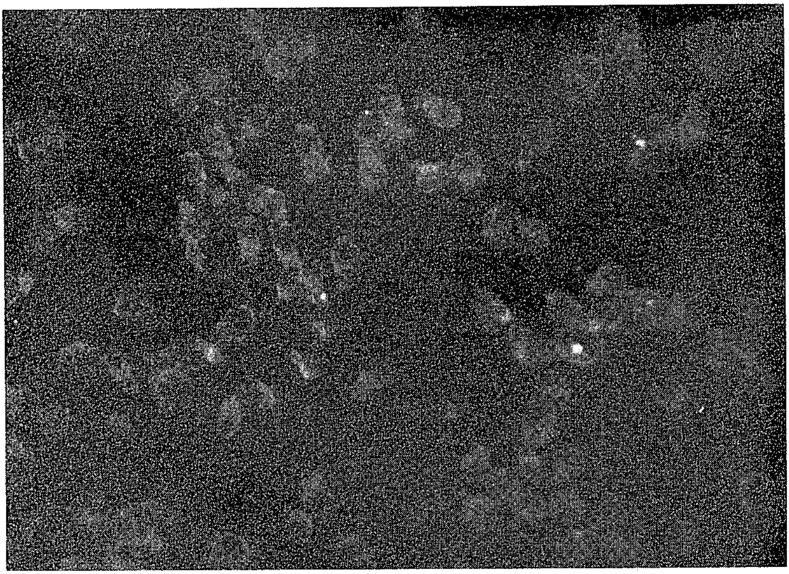
Fixation and FA reading

The growth medium is removed after a 24-hour incubation period, and the slides are then rinsed once in phosphate-buffered saline, once in acetone at room temperature, and then fixed for 1 minute in acetone at −20°C. The FA staining (Goldwasser & Kissling, 1958) is performed as described in chapter 6 (page 73) with conjugate prepared according to standard procedures (see chapter 6, page 80), and treated twice with normal BHK-21/S13 cells. The slides are examined with a fluorescent microscope using suitable exciter and barrier filters, the illumination being supplied by an Osram HBO 200 high-pressure mercury vapour lamp. Twenty low-power (160 ×) microscopic fields are observed for each of the 8 Lab-Tek dilution chambers, and the number of fields containing fluorescing cells are tabulated. Of the 20 fields observed in the challenge virus control slides, 18–20 contain fluorescing cells (Fig. 2). A reduction of 50% or more in the number of fields with fluorescing cells (Fig. 3) indicates the presence of neutralizing antibody in the test serum.

Results

We have compared RFFIT results with those obtained in the standard mouse VN index test (see chapter 8, page 94). There was over

FIG. 3: NON-INFECTED BHK-21/S13 CELLS (ANTIBODY PRESENT)



95% agreement between the results obtained by the two methods, with approximately 5% of the sera negative by the VN test but positive by the RFFIT. No antibody has been detected in sera of unvaccinated persons. Moreover, the levels of VN antibody determined by the RFFIT are identical with those found in the mouse neutralization index test.

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THE IMMUNOPEROXIDASE REACTION FOR DEMONSTRATION OF RABIES VIRUS

P. ATANASIU[1]

The introduction into histochemistry of enzyme-labelled antibodies for the localization of antigens (Nakane & Pierce, 1966) was followed by the development of the immunoperoxidase reaction for use in virology (Kurstak et al., 1969 ; Wicker & Avrameas, 1969). This method has now been developed into a diagnostic test for the detection of rabies antigen in cells.

The procedures described below include the purification of rabies antibody by means of immunoadsorption, the preparation of antirabies-peroxidase conjugate, and the direct and indirect staining techniques. The results obtained are also discussed.

Preparation of immunoadsorbed rabies antibody

Mice aged 4 days are inoculated intracerebrally with 100 LD_{50} of the Pasteur strain of rabies virus. The brains are harvested 5 days later using an aspiration syringe connected to a vacuum pump. After the brains have been homogenized, the homogenate is diluted with an equal quantity of distilled water. The mixture is next centrifuged for 10 minutes at 1 500 *g* and a protein determination is carried out on 10 ml of the supernatant liquid by the Biuret method. Bovine serum albumin is then added in the proportion 4 parts of albumin to 1 part of protein antigen. The whole is mixed with 40 ml of a buffered acetic acid solution, pH 5 (0.2M acetic acid plus 0.2M sodium acetate solution), and 8 ml of a 2.5% glutaraldehyde solution are added. Two hours later, the gel obtained is washed several times with phosphate-buffered saline, pH 7.4 (0.15M sodium chloride solution containing 0.01M phosphate buffer) and 10 ml of hyperimmune antirabies serum are then added. After shaking for 1 hour at room temperature to allow immunoadsorption to take place, the gel is again washed several times with phosphate-buffered saline.

The immunoadsorbed antibody is recovered from the gel by elution with an acid buffer, pH 2.8, containing 0.2N hydrochloric acid and 0.2M glycine. The total quantity of protein obtained is determined. After neutralization, the solution is dialysed overnight and then concentrated (Avrameas & Ternynck, 1969); the final concentration should be 5–7 mg of purified antibody per ml.

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[1] Head, Rabies and Rhabdovirus Research Laboratory, Pasteur Institute, Paris, France.

Conjugation of rabies antibody with peroxidase

The pH of the solution of purified rabies antibody is adjusted to 6.8 by addition of a 0.1M solution of potassium phosphate (K_2HPO_4). For each 1 ml of solution containing 5–7 mg of purified antibody, 10–14 mg of peroxidase[1] are added, followed by 0.05 ml of 1% glutaraldehyde. The mixture is shaken for 2 hours at room temperature and then dialysed overnight against physiological saline, pH 7.4. The next day the solution is centrifuged for 15 minutes at 1 500–2 000 *g*. The conjugate so obtained is diluted 1 : 10 before use (Avrameas & Ternynck, 1969).

Direct staining

The freshly received material (brain smears of fixed or street virus, frozen sections, cell cultures containing the virus, etc.) is immediately fixed by immersion for 10 minutes in cold acetone (−20°C). The dried preparation, on slides or coverslips, is covered with the peroxidase-antibody conjugate diluted 1 : 10 with physiological saline and left in a humid atmosphere for 3 hours at room temperature. It is then washed with distilled water and left for 5–10 minutes in contact with a solution of 3,3′,4,4′-tetra-aminobiphenyl tetrahydrochloride (0.5 g/litre), buffered to pH 7.4 with 0.1M tris. After washing again, it is mounted in Elvanol[2] and examined under a light microscope.

Indirect staining

This can be done on smears, frozen sections 3–5 µm thick, or infected cell cultures, after fixation or drying. The preparation is covered with an antirabies serum (preferably one prepared from the rabies nucleocapsid in rabbits), diluted 1 : 100, or with a human serum diluted 1 : 5 or 1 : 10. Coons barbital buffer, pH 7.2,[3] is used for making all the dilutions and for washing. The preparation is left in contact with the serum for 30 minutes at room temperature and is then washed for 15 minutes with diluted Coons buffer and rinsed with distilled water.

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[1] A suitable grade is Type VI (RZ approx. 3.0), obtainable from the Sigma Chemical Company, P.O. Box 14508, St. Louis, Miss., USA.

[2] A polyvinyl alcohol, which can be prepared by the method of Rodriguez & Deinhardt (1960). Also obtainable from E. I. Du Pont de Nemours & Co., Inc., Wulmington 98, Del., USA.

[3] *Stock solution* : sodium chloride, 85 g ; barbital sodium, 20.6 g ; 1.0N hydrochloric

sheep serum against rabbit or human immunoglobulin,[1] diluted with Coons buffer in accordance with the instructions. The conjugate is left in contact with the preparation for 60 minutes at room temperature. After washing, the peroxidase activity is determined by leaving for 5–10 minutes in contact with the same buffered tetra-aminobiphenyl solution used in the direct method. The preparation is washed again, mounted in Elvanol, and examined under the light microscope.

Both positive and negative controls are necessary for each staining method (direct and indirect).

Results

In brain smears containing fixed virus, the rabies antigen may appear in the form of small or large particles, or as a powdery mass. On the other hand, in the case of street rabies, the antigen appears as large, yellow-brown particles, which are darker in colour towards the edges (see Plate I F, facing page 72). The same difference is observed using the fluorescent antibody technique (see Plate II, facing page 76).

In rare cases, it has been possible to discern the internal structure of the larger particles, in the form of darker condensations.

In cell cultures and organ sections, the antigen is seen at a very early stage in the cytoplasm and later distributed throughout a group of contiguous cells, giving the appearance of a focus. Its size varies from the limit of visibility up to 10 μm. It has the same dark yellow-brown colour described above.

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Nakane, P. K. & Pierce, G. B. (1966) *J. Histochem. Cytochem.*, **14**, 929

Rodriguez, G. & Deinhardt, F. (1960) *Virology*, **12**, 316

Wicker, R. & Avrameas, S. (1969) *J. gen. Virol.*, **4**, 465

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[1] This is obtainable from Production S.A., Institut Pasteur, 25 rue du Docteur Roux F 75 Paris 15e, France.

APPENDIX 7

ADDRESSES OF WHO REFERENCE CENTRES FOR RABIES

International Reference Centres

Institut Pasteur, 25, rue du Docteur Roux, F 75 Paris 15^{e}, France

Pasteur Institute of Southern India, Coonoor 3, India

Institute of Poliomyelitis and Viral Encephalitides, Academy of Medical
Sciences of the USSR, Kievskoe Šosse 27 km, Moscow V. 27, USSR

The Wistar Institute, Thirty-sixth Street at Spruce, Philadelphia, Pa 19104,
USA

Regional Reference Centre for the Americas

Center for Disease Control, Public Health Service, P.O. Box 363,
Lawrenceville, Ga 30245, USA

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